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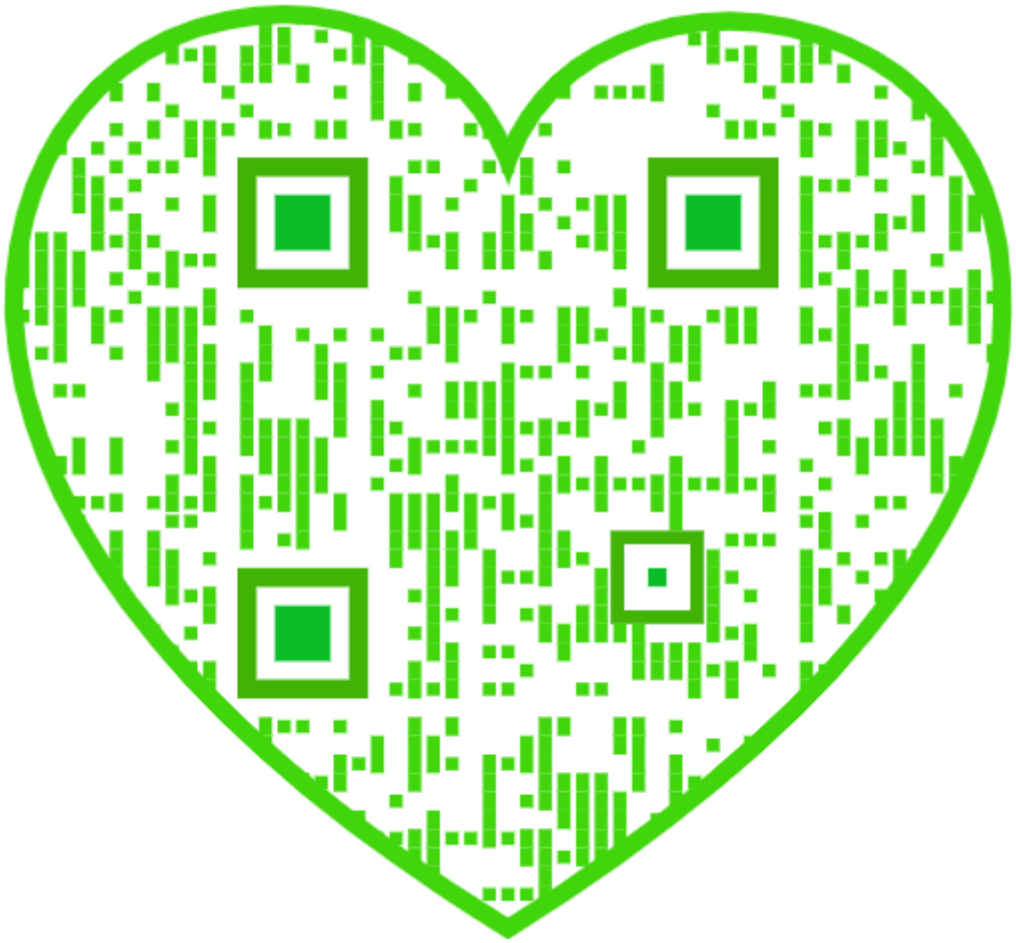
LABORATORY TECHNIQUES IN RABIES



LABORATORY TECHNIQUES IN RABIES

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PREFACE

The first edition of Laboratory Techniques in Rabies *was published in 1954 and was well received by laboratories concerned with rabies problems. Numerous advances in laboratory methods applicable to rabies have since taken place, and the time is now considered ripe for a second edition of this manual. Nearly all the new diagnostic procedures and methods of vaccine production described are suitable for use in the rather modestly equipped and staffed rabies laboratories found in most countries of the world, especially in developing countries.*

During the ten years that have elapsed since publication of the first edition, the procedures described in the manual have been kept under constant review at meetings of research groups and of the WHO Expert Committee on Rabies. The fifth report of the Committee [1] *has recently been published and forms an essential companion piece to this monograph.*

In bringing the manual up to date, a certain number of changes have been made in the contents. A chapter has been added on " The Laboratory in the Diagnosis and Prevention of Rabies " to give some guidance on the relative advantages and difficulties of the different procedures described for the diagnosis of rabies, and on the production and potency testing of biological products. In another new chapter, the recently developed and highly successful application of the fluorescent-antibody technique to the diagnosis of rabies is described in detail. A description of the production of vaccines in tissue culture has been included, because such vaccines have already come into use in veterinary medicine, and will undoubtedly find application in human beings after completion of the trials now in progress as part of the co-ordinated research being carried out under WHO auspices. The production of freeze-dried rabies vaccine using brains of suckling rats or mice to avoid the paralytic factor present in adult animal brains is also described. Freeze drying has now been successfully applied to other rabies vaccines. Production of duck-embryo vaccine, widely employed in the USA, unfortunately could not be included, but the procedure is analogous to the one employed for chicken-embryo vaccines, with the addition of inactivation by betapropiolactone. The chapter on breeding and care of laboratory animals included in the first edition has been dropped because excellent publications on this specialized subject have since appeared.

The following paragraphs from the Foreword *to the first edition apply equally to the second :*

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1966, **321**.

" The manual is not intended as an exhaustive treatise : its scope has been purposely limited to one or two procedures in each of the major divisions of laboratory techniques in rabies. The contributors were requested to select and present procedures based on their own experience which would be dependable and practicable without sacrificing necessary minimal standards, but which at the same time could be adapted to the limited facilities and personnel of many rabies laboratories in different parts of the world. The techniques were selected also with a view to encouraging and facilitating uniform methods which would permit of a more valid comparison of results obtained in different laboratories.

"Work with viruses is a highly developed discipline which permits of little latitude if reproducible results are to be obtained. However, it is to be expected that the opinions of individual workers on techniques will differ with respect to details. The techniques recommended here have been prepared for particular application in rabies work, although it is evident that some of them, such as the serum-virus neutralization test and the mouse inoculation test, are readily applicable, perhaps with slight modifications, to other virus diseases. It will be noted further that in describing the various techniques a rational and systematic approach has been stressed by the contributors so that errors which might otherwise nullify excellent work may be avoided."

The World Health Organization wishes to record its gratitude to all the contributors to this manual for the time and care they have devoted to the preparation of their chapters.

PART I

LABORATORY DIAGNOSIS

CHAPTER 1

THE LABORATORY IN THE DIAGNOSIS AND PREVENTION OF RABIES

MARTIN M. KAPLAN [1]

Few diseases cause as much anxiety as does rabies ; this applies equally to the exposed individuals and to the health authorities responsible for its prevention and control. The laboratory occupies a central place in meeting the threat of rabies because upon its verdict often depend both the decision whether or not to proceed with a long and painful course of treatment and the decision on the need to institute elaborate measures for controlling an epizootic in a community. The laboratory must also provide the necessary assurance that the biological products used for treatment and prevention in man and animals are efficient and safe.

The succeeding chapters in this manual describe selected but multiple methods for arriving at a diagnosis in the laboratory and for determining the acceptability of biological products in rabies prophylaxis. Most laboratory workers can decide for themselves whether one or other of the techniques given is within their competence, but often they are not aware of certain pitfalls and the limitations of particular methods. Also, a choice of procedures can ease the work and provide a decisive answer quickly. These considerations are partially covered in the relevant sections of this manual ; here they are reviewed and evaluated more extensively to serve as a simplified guide for selecting procedures and for interpreting the results obtained.

The institution of treatment measures in exposed individuals, as recommended in the reports of the WHO Expert Committee on Rabies, should never await the results of laboratory diagnosis. A laboratory diagnosis may be delayed for a variety of reasons and early treatment, both local and systemic, can be a critical factor in saving the life of the patient.

A laboratory report should be as clear and unequivocal as possible, and should stipulate exactly the procedures used. A positive test by any one of several procedures cancels negative reactions to the others. Thus, if a fluorescent-antibody test is positive and the examination for Negri bodies or attempted isolation of the rabies virus by animal inoculation gives negative results, the report should read " positive ". Where a doubtful result is obtained in any single test, recourse to the other tests available

[1] Chief, Veterinary Public Health, Division of Communicable Diseases, WHO, Geneva, Switzerland.

is essential in order to arrive at a definitive conclusion. Until this conclusion is reached, treatment should be continued as recommended by the WHO Expert Committee. Despite a completely negative laboratory report, circumstances may occasionally justify the initiation or continuation of treatment by the physician, e.g., suspicious clinical signs in the animal, or an unprovoked attack by an animal in an area where rabies is enzootic. Considerable experience has demonstrated, however, that a complete set of negative results obtained in a reliable diagnostic laboratory can be accepted with confidence, and that the treatment can be terminated or modified at that point.

A. Diagnostic Procedures

1. Histopathology (chapters 2, 3 & 4)

The quickest and easiest procedure is the use of a suitable dye for the detection of Negri bodies. The Sellers, Giemsa and Mann stains are widely used and all of them are satisfactory. The Sellers stain combines simplicity with easy identification of the Negri bodies. With all the stains, care must be taken to distinguish the Negri bodies from inclusion bodies of other viral diseases, and from artifacts. A competent laboratory worker can do this only after extensive training and supervision. A positive diagnosis based on the detection of Negri bodies can be made within half an hour after receipt of a specimen. If the reading is negative, the fluorescent-antibody technique (chapter 5) should be employed if available and the mouse inoculation test started (chapter 6). Rapid histological techniques are now available using frozen sections, but both this method and the classical method using paraffin-embedded sections are rapidly becoming obsolete now that rapid diagnosis can be achieved by means of the fluorescent-antibody technique ; they have value, however, in softened brain material and for research purposes where permanent mounts are desired.

The finding of specific inclusion bodies or rabies antigen (fluorescent-antibody technique) in the submaxillary salivary glands is of particular importance for treatment purposes. This examination should always be carried out because "animals do not bite with their brains" : the finding of rabies virus in the brain of an animal with none in the salivary glands removes some of the anxiety attendant upon treating an exposed person, although it does not entirely exclude the possibility that virus was being excreted at the time of the bite.

2. Animal inoculation (chapter 6)

The suckling mouse is the most susceptible animal for the inoculation test. While street virus has a variable incubation period of 7 to 18 days,

and occasionally longer, rabies antigen can sometimes be detected as early as the second day following inoculation if the fluorescent-antibody test is then applied. It is preferable to use 15 to 20 mice sucklings or mice 3-5 weeks old for the inoculation test, so that 1 or 2 mice can be sacrificed each day, starting on the second day after inoculation ; this permits the use of the fluorescent-antibody technique and staining for Negri bodies for early detection of the virus. Negri bodies can sometimes be demonstrated as early as the third or fourth day after inoculation, before clinical signs begin.

Mouse inoculations should be employed in all cases where Negri bodies are not found, because in some laboratories positive results have been obtained by the mouse inoculation test in up to 20% of cases where Negri bodies were reported absent. In very experienced laboratories, the discrepancy may be much lower (less than 5%), and agreement between the fluorescent-antibody test and the mouse inoculation test approaches 100%.

3. Fluorescent-antibody (FA) test (chapter 5)

When properly performed, the " direct " FA test takes the lead over all others for speed and accuracy combined. Readings can be obtained within half an hour following receipt of the specimen, although for routine purposes a period of 2-4 hours or more is allowed for the fixation in cold acetone. The " indirect " FA test is useful for screening serum specimens for antibody, but this requires that the specific antiglobulin of the species concerned be conjugated with fluorescein isothiocyanate. Special FA equipment that can be used with ordinary light microscopes can now be purchased separately, so that the cost of adopting the FA technique has been much reduced and is now within the reach of most laboratories.

Apart from a satisfactory microscope, the two main requirements for success in using this technique are a well-trained technician, and conjugated serum of good quality. Intensive training in a good laboratory for a minimum of several weeks is necessary for a careful worker, but frequent confirmatory checks of positive and negative specimens using mouse inoculations should be carried out during the first year of operations, or until complete confidence is gained as to the accuracy of the technique. After one year's experience most laboratories find over 99% agreement between the FA test and the mouse inoculation test (correlation of both positives and negatives). In the first and second year of operations, however, some laboratories may miss up to 10% or even 20% of the positives with the FA technique, and for this reason the mouse-inoculation test must be used during this period on all FA-negative specimens.

Small laboratories should not attempt to prepare their own conjugated antirabies serum (or gamma globulin) because of the difficulties involved ; they should try to obtain the small supply usually required from a large

central laboratory, or from reliable commercial sources. All batches of conjugated sera should be carefully tested on known positive and negative specimens, and *each* specimen tested should have a control as described in chapter 5. Only then can the diagnosis be accepted with complete confidence.

4. Serum-virus neutralization test (chapter 7)

This is the most accurate procedure for determining the specificity of the rabies virus. It should be combined with the fluorescent-antibody test for these purposes. In addition, the serum-virus neutralization test can be used for detecting the presence and amount of antibody in a serum ; the serum-dilution constant-virus technique is the preferred one for such tests. Readings of the test require 7-14 days so that it cannot be used for rapid diagnosis.

5. Complement-fixation test

This test has limited usefulness for diagnosis because of its unreliability as compared with the other tests described. It is no longer widely used, although a few laboratories still consider it to be of value.

6. Other tests

Rapidly advancing research in tissue culture may well produce practicable methods for direct isolation of the rabies virus, and serum-virus neutralization tests in tissue culture. At the present time, such techniques are in the experimental stage.

B. Production of Vaccine and Serum (Parts II and IV)

1. Vaccine

The selection of a particular type of vaccine for production and use often poses a dilemma. This can be resolved if one keeps in mind the main purpose of a rabies vaccine : to protect against rabies. All other considerations are secondary, although admittedly important at times, e.g., paralytic accidents. The first principle is never to tolerate vaccines of low potency, regardless of other possible virtues (stability, absence of paralytic factor). The laboratory should aim to produce the highest antigenic content in the lowest possible quantity of extraneous protein material. Improved vaccines will undoubtedly be developed in the future using purified viral protein, but for some years to come we shall have to depend upon the nervous tissue and avian embryo vaccines now available. Tissue-culture

vaccines are already in use in veterinary medicine and should shortly become available for human use.

The two main types of nervous tissue vaccine are Semple and Fermi, using heat and phenol for complete or partial inactivation respectively (the Fermi-type vaccine contains a slight residual amount of living fixed virus). Potent suckling-mouse-brain and suckling-rat-brain vaccines have been developed and these have the advantage of being practically devoid of the paralytic factor. Many other types of vaccine are produced and widely used, e.g., ultraviolet-inactivated ; duck-embryo (propiolactone-inactivated) ; vaccines inactivated with formaldehyde, chloroform (Kelser) or ether (Hempt) ; and dilutions of living fixed virus (Högyes, Pasteur). All these vaccines can now be produced in freeze-dried form, which is a great advantage from the standpoint of stability. Semple and Fermi type vaccines are the easiest to produce. Highly potent ultraviolet-inactivated vaccine is not difficult to produce once the apparatus has been properly adjusted. Difficulties are sometimes experienced in producing potent duck-embryo vaccine, as well as some of the other types (formol-, ether- and chloroform-inactivated). The Högyes and Pasteur type vaccines are no longer widely employed.

Regardless of the type of vaccine produced, in addition to a potency test all batches of vaccine should be subjected to the safety tests recommended, including the absence of contaminants and, with the Fermi-type vaccine, assurance of only a low residual amount of live virus (see chapters 10, 11, 12, 13 & 14).

2. Serum

Horses, mules, sheep, cattle and rabbits — particularly the first three — are commonly employed for the preparation of antirabies serum, but in up to 20 % of cases adverse reactions to heterologous serum, often of a serious nature, are encountered in man. Partial purification of the serum or the preparation of the globulin fractions slightly lessens the frequency of such adverse reactions. The production of specific antirabies human gamma-globulin is still in the experimental stage but it appears to be a practicable procedure, although expensive ; if sufficient stocks can be produced, for example by the plasmaphoresis technique, this preparation would avoid completely the adverse serum reactions resulting from heterologous serum.

C. Potency Tests (Parts III & IV)

1. Vaccine

Experience has shown that all batches of vaccine, regardless of the technique used, must be tested for potency, because batches vary in potency

even though the same method of production is employed routinely. The appropriate potency test described in Part III should be used. The Habel test and its modifications, especially the NIH antigen-extinction test using a reference vaccine (chapter 18), are time-proven for all vaccines except the living attenuated vaccines produced in chicken embryos for veterinary use. The latter require a different type of test (chapters 19 & 20). The rationale of potency tests is summarized in chapter 15.

It should be kept in mind that too great a quantitative significance should not be given to the protection index obtained either in the Habel potency test or in the NIH antigen-extinction test. In both cases, the index is calculated using the Reed & Muench method and it should be considered as an approximation rather than as strictly quantitative. For example, a vaccine calculated to have an index of 12 000 in the Habel test cannot be said to be twelve times as potent as one found to have an index of 1000, the minimum acceptable figure for potency. Both the Habel test and the NIH test are designed to establish minimum rather then absolute values for potency.

The stability of liquid-suspension vaccines is very variable. Unrefrigerated storage in hot climates or the freezing of a liquid phenolized suspension can completely destroy the potency of a vaccine. Dried vaccine containing too high a residual moisture content (over 2 %) is not usually stable. For vaccines produced and handled under certain local conditions, e.g., hot climates, frequent checks are necessary to determine that potency has been retained. The routine expiry dating of a vaccine cannot be depended upon where decentralization of treatment is employed, e.g., retention of vaccine stocks in rural treatment centres. The central laboratory should therefore recall specimens periodically from field stations to ensure that loss of potency has not occurred under their system of decentralization.

2. Serum

The potency test described in chapter 22 is essential in order to determine whether a particular batch of serum, or its globulin preparation, is acceptable with respect to its content of antibody, based on international units.

* * *

The success or failure of rabies prophylaxis and control depends upon the laboratory. The exposed individual and the health authorities are best served through strict laboratory disciplines and a constant awareness of the possible weak points and limitations of the laboratory techniques used.

SHIPMENT OF SPECIMENS, AND TECHNIQUES FOR PREPARATION OF ANIMAL TISSUES

ERNEST S. TIERKEL [1]

The animal suspected of being rabid should be captured and confined under observation for 10 days, if possible, and the disease should be allowed to progress until fatal termination. The premature killing of such animals will reduce the accuracy of the laboratory diagnosis, since the development of Negri bodies in the brain is directly related to the length of clinical illness in rabies. If circumstances necessitate the destruction of the animal, it should be shot through the heart, because shooting through the head will damage the brain and render it less useful for diagnosis. The use of chemical poisons is not recommended, since they may interfere with subsequent animal-inoculation tests.

Packing and Shipment of the Specimen

After decapitation of the animal in the field, the head should be cooled down promptly and kept cold. Wherever possible it should be delivered by messenger. If no messenger service is available, the head should be packed for shipment by express freight, via rail, air, or road. It should be put into a tin or other suitable watertight metal container, which should be closed tightly. This in turn should be put into a larger watertight metal container, cracked ice being packed between the inner and outer container (see Fig. 1 and 2). The package should be clearly labelled and shipped to the laboratory with utmost dispatch.

NOTE : Although freezing the specimen and shipping it frozen in dry ice (solid carbon dioxide) or in nitrogen flasks will preserve the virus, quick microscopic examination may be delayed because of the time necessary for the head to thaw. Frozen portions of brain and salivary glands are easier to handle in the laboratory than are frozen entire heads. Immediately upon thawing, the tissues should be made ready for direct microscopic examination for Negri bodies, for the fluorescent antibody test, or for the mouse inoculation test.

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[1] Deputy Director, Health Service, Office of Technical Cooperation and Research, Agency for International Development, Washington, D.C., USA.

FIG. 1. DOUBLE CONTAINER WITH ICE FOR PACKING SPECIMENS

By courtesy of United States Department of Health, Education and Welfare, Public Health Service, Communicable Disease Center (US DHEW-PHS-CDC)

FIG. 2. CONTAINER COVERED AND LABELLED

By courtesy of US DHEW-PHS-CDC

The following information is desirable when animal heads are received for examination: the species and breed of the animal; whether it was in contact with other animals; whether the animal died or was killed, and, if the latter, the means used for destroying it; whether the animal was confined and observed for an appropriate time before death, and, if so, for how long; symptoms of rabies, if any; history of vaccination against rabies.

Removal of Animal Brain

All precautions, including careful operative technique and protection of the hands with heavy rubber autopsy-gloves, should be taken to prevent infection of persons opening animal heads.

The head should be held firmly on a solid table; proper immobilization may be effected by grasping it with a lion-jaw type of bone-holding forceps firmly applied to the maxilla. Improvised mechanical devices such as a carpenter's vice have often also been found effective.

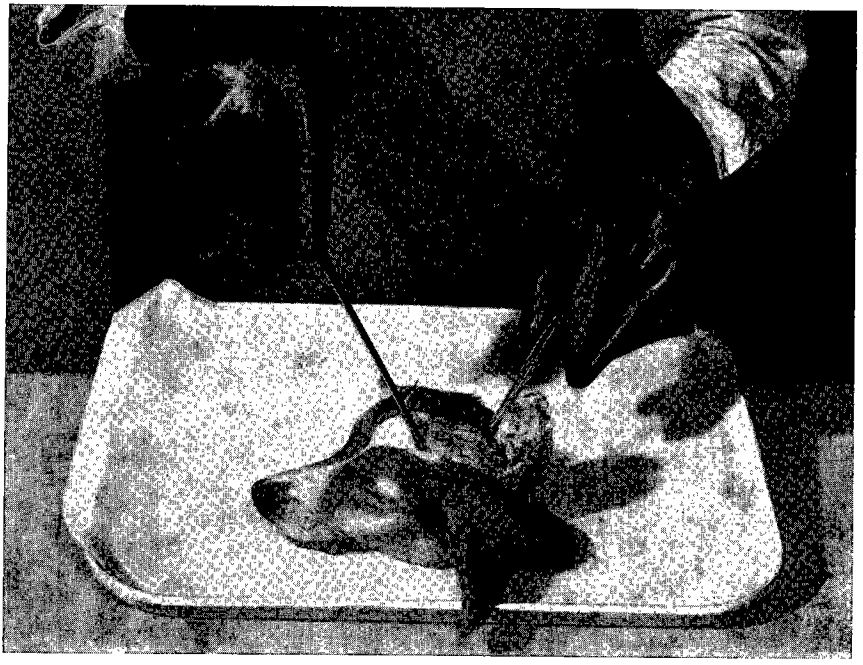
FIG. 3. INITIAL SKIN INCISION ALONG MIDLINE OF DOG'S HEAD

By courtesy of US DHEW-PHS-CDC

FIG. 4. USE OF BONE-SAW FOR MAKING TRANSVERSE ANTERIOR INCISION IN FRONTAL BONES JUST ABOVE THE EYES

By courtesy of US DHEW-PHS-CDC

FIG. 5. CALVARIA REFLECTED



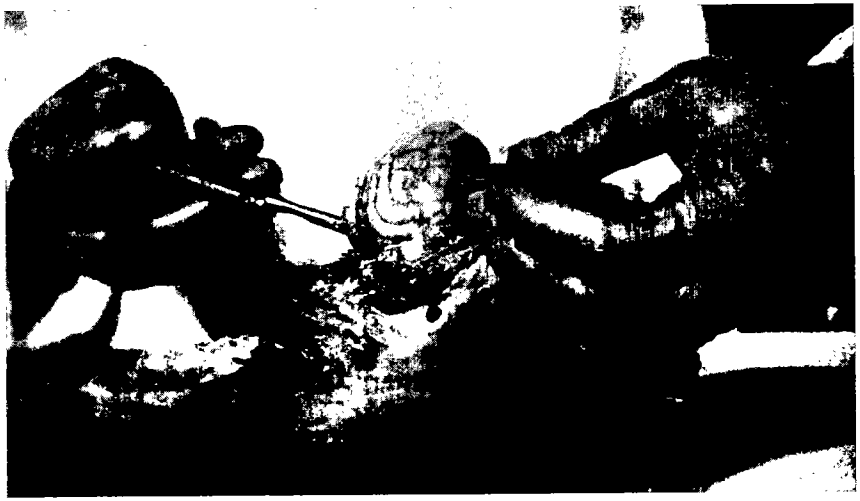
By courtesy of US DHEW-PHS-CDC

With section knife or large scalpel a midline incision is made through the skin, fasciae, and muscles of the cranium, beginning anteriorly just above the level of the eyes and extending posteriorly to the base of the skull (see Fig. 3) The skin, fasciae, and temporal muscles are then dissected away from the cranium and reflected laterally exposing the bone. The calvaria is then removed by means of a saw, bone-chisel, or butcher's cleaver. The sawing method is preferred by most laboratories. In this procedure a sterile surgical bone-saw is used to incise the bone of the skull on each side, beginning at the foramen magnum and then sawing anteriorly to the frontal bones. The longitudinal cuts are then joined by a transverse incision through the frontal plate just above the eyes (see Fig. 4). The calvaria is then lifted off with the aid of bone-cutting forceps and/or a bone-chisel (see Fig. 5).

With a fresh set of sterile instruments, the brain is now removed from the cranium. The meninges and the tentorium cerebelli which separates the cerebrum and cerebellum are dissected away with rat-tooth thumb forceps and either a scalpel or sharp-pointed scissors. Next, with a scalpel or scissors reaching back into the posterior portion of the brain, the brain is severed from its site by cutting into the medulla, cranial nerves, and anterior extension of the thalamus. The entire brain is then lifted out of the cranium on to a paper picnic-plate or a large Petri dish (see Fig. 6).

If the body of the animal is available and no typical Negri bodies are found in the rapidly stained films, make a general post-mortem examination in an attempt to determine the reason for the illness or unusual behaviour

FIG. 6. REMOVAL OF ENTIRE BRAIN

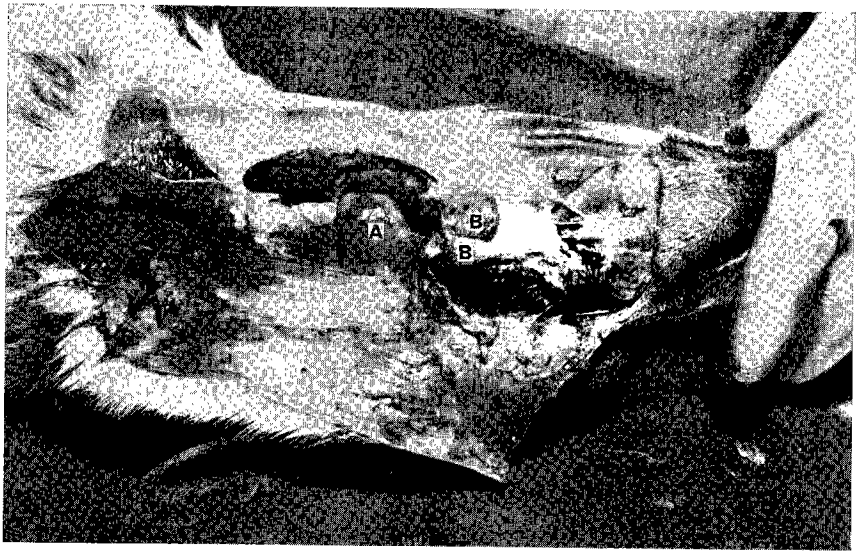


By courtesy of US DHEW-PHS-CDC

of the animal. Wrap the remains in paper and ensure their incineration. Wash the table with 10% cresol solution. Sponge the gloves in the same kind of solution before removing them from the hands, and then sterilize them, with the instruments, by boiling.

It is always advisable to record the gross appearance of the brain, indicating especially the state of preservation, the presence or absence of

FIG. 7. SKIN REFLECTED AND SUBCUTANEOUS TISSUE REMOVED, EXPOSING SUBMAXILLARY SALIVARY GLAND



A Submaxillary salivary gland
B Submaxillary lymph glands

adhesions and exudate, and whether the blood-vessels are injected. There are no gross pathological changes that can be regarded as diagnostic of rabies.

Even in the field, precautions should be taken to prevent bacterial contamination of the animal brain by the use of sterile instruments in brain removal and dissection.

Removal of Salivary Glands

The examination of the salivary glands for the presence of virus is of obvious value in providing definitive evidence of whether or not a bite has entailed a risk. Presence of the infection in the central nervous system does not necessarily indicate infective saliva.

If virus is present in the salivary glands, the submaxillary (mandibular) glands will contain the most virus. For removal of the submaxillary salivary glands, the head is turned over so that the ventral aspect is facing upward. With an autopsy knife or scalpel, a midline incision is made in the skin covering the area between the rami of the mandible. The incision should begin at the mandibular lip and extend posteriorly to the neck. The skin is then reflected laterally, exposing the muscles and superficial soft tissues of the lower jaw.

FIG. 8. LEFT SUBMAXILLARY SALIVARY GLAND COMPLETELY EXCISED

A Submaxillary salivary gland
B Submaxillary lymph glands

The submaxillary salivary gland on each side is situated quite superficially and after reflecting the skin back it can be seen in the area of the posterior border of the mandible behind and below the superficial submaxillary lymph glands, with which the salivary glands should not be confused (see Fig. 7 and 8). The submaxillary salivary gland is elliptical in shape, about 5 cm long and 3 cm wide ; it is rounded in outline, greyish-yellow or orange in colour, and covered by a fibrous capsule.

With a fresh sterile scalpel and rat-tooth thumb forceps, the gland on each side is dissected away from its surrounding tissues and placed in a Petri dish.

A small portion of each salivary gland is cut with sterile scissors and the portions are pooled in a single mortar for preparation of the salivary-gland suspension for the mouse inoculation test (see chapter 6, page 70). The test pieces should be weighed before emulsification for calculation of the concentration of the tissue suspension (see page 49 for gasserian ganglion).

Glycerolated Specimens

When facilities for animal inoculation of Negri-negative or doubtful animal brains suspected of rabies infection are not at hand, it is possible to ship portions of brain or salivary gland to a laboratory that performs animal inoculation tests.

A solution of sterile 50% glycerol-saline is prepared by adding equal parts of chemically pure glycerol to physiological salt solution. This solution may then be placed in small bottles or jars with screw tops, autoclaved, and stored at room temperature.

Fairly large portions of brain or salivary gland, at least about the size of a mouse brain (0.3 g or more), should be selected in order to give the laboratory worker sufficient material with which to work. Portions of the brain should include parts of the hippocampus, cerebellum, and cerebral cortex from each side. A portion of the medulla or brain stem should also be included. Place the tissue pieces in a bottle of prepared 50% glycerol-saline. Rabies virus, if present, will be preserved during shipment, and no refrigeration is required.

Glycerolated portions of brain do not usually produce satisfactory impressions on a slide unless washed thoroughly in saline, since it is difficult to make the glycerolated brain adhere to a slide. This is true whether the tissue impression is to be used for Negri body staining or for the fluorescent antibody test. For this reason, if further examination of brain smears on the questionable specimen is desired, smears may first be made from the unpreserved brain and stained, and the slides included in the shipment along with the glycerolated brain. Alternatively, brain smears may be made and the *unstained* slides plunged immediately into chemically pure methanol (acetone-free) for fixation, and then removed and dried at room temperature without blotting. These unstained, fixed smears can then be stained at the laboratory to which they are referred.

Upon their receipt in the laboratory, glycerolated tissue specimens should be immediately removed from the shipping bottle and placed in a sterile Petri dish. The pieces of tissue are then thoroughly washed in

physiological saline solution by continual addition of the solution, gentle rotary agitation of the Petri dish, and discarding of the liquid. This is repeated several times. At this point it is as well to attempt a smear preparation of the washed brain-tissue specimens on a slide for Negri-body staining and microscopic examination. The quality of the smear will depend on the amount of glycerol washed from the brain tissue. The remaining specimens are now ready for emulsification and mouse inoculation. For grinding salivary glands in a mortar a small amount of sterile sand or alundum is essential, but these materials are superfluous for making brain suspensions.

Note : It is advisable to retain in glycerol solution some unused portions of all animal-tissue specimens until the animal inoculation test either has been reported positive for rabies, or has run its course (at least 21 days) and has been reported negative for rabies.

RAPID MICROSCOPIC EXAMINATION FOR NEGRI BODIES AND PREPARATION OF SPECIMENS FOR BIOLOGICAL TEST

ERNEST S. TIERKEL [1]

The techniques employed in the laboratory diagnosis of rabies should embrace optimum conditions of accuracy, speed, and economy. The method employing the microscopic examination for Negri bodies, using the simple application of brain tissue to a slide and Sellers' technique for staining (see page 34), has been proved to fulfil these requirements.

It has been found that Negri bodies, when present, are most readily demonstrated in Ammon's horn (hippocampus major) of the brain and also in the pyramidal cells of the cerebral cortex and Purkinje's cells of the cerebellum ; they are found to a much more limited extent in the neurons of the thalamus, pons, medulla, spinal cord, and sensory ganglia.

Dissection of the Brain

A very simple operation is required to expose Ammon's horn, which is generally the best area for demonstration of Negri bodies in most species of rabid animals. With a pair of sterile-scissors, a longitudinal incision is made into the dorsal surface of each cerebral hemisphere, about 2 cm lateral to the longitudinal fissure or midline of the brain (see Fig. 1). The incision is made from the region of the occipital pole of the hemisphere and is extended forward for 3-5 cm and downward through the grey matter, and then completely through the white matter until a narrow space, the lateral ventricle, is reached. The opening is then widened by spreading the incised hemisphere, and Ammon's horn will be revealed as a semi-cylindrical, white, glistening body bulging laterally from the ventricle floor (see Fig. 2 and 3). It has a spiral contour and, on cross-section, a characteristically rolled surface.

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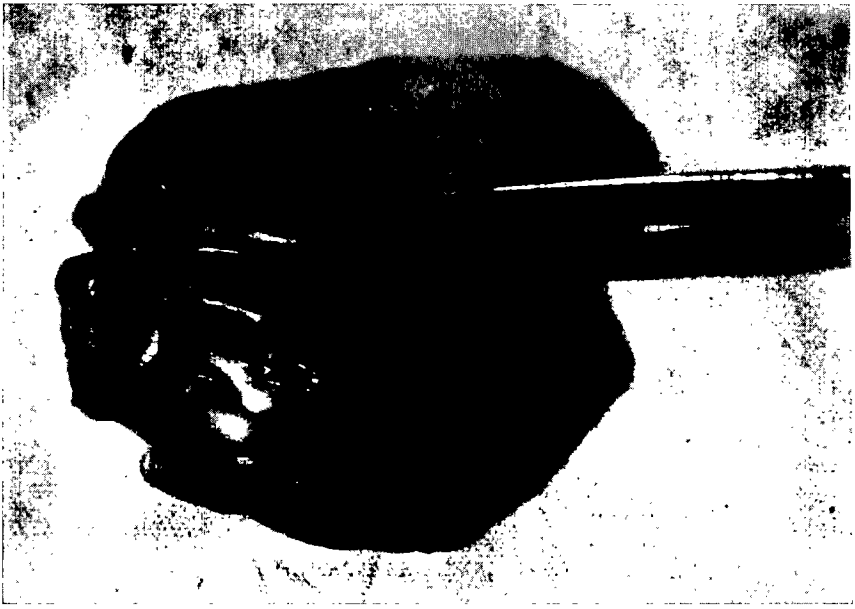
[1] Deputy Director, Health Service, Office of Technical Cooperation and Research, Agency for International Development, Washington, D.C., USA.

FIG. 1. SITE OF INCISION FOR LOCATING AMMON'S HORN



By courtesy of United States Department of Health, Education and Welfare, Public Health Service, Communicable Disease Center (US DHEW-PHS-CDC)

Preparation of Slide

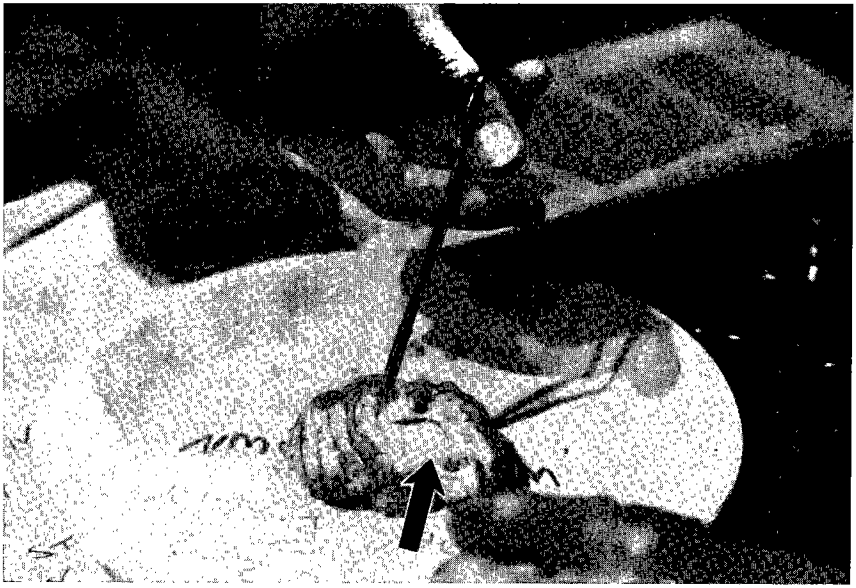
Slides should be made first from Ammon's horn, then from the cerebral cortex, and finally from the cerebellum. Samples (at least six) should be taken from these three areas on each side of the brain and examined microscopically before the brain is reported Negri-negative. It is always wise to select another area from each hippocampus for good measure.

The following three methods of applying fresh brain tissue to slides can be recommended :

Impression method

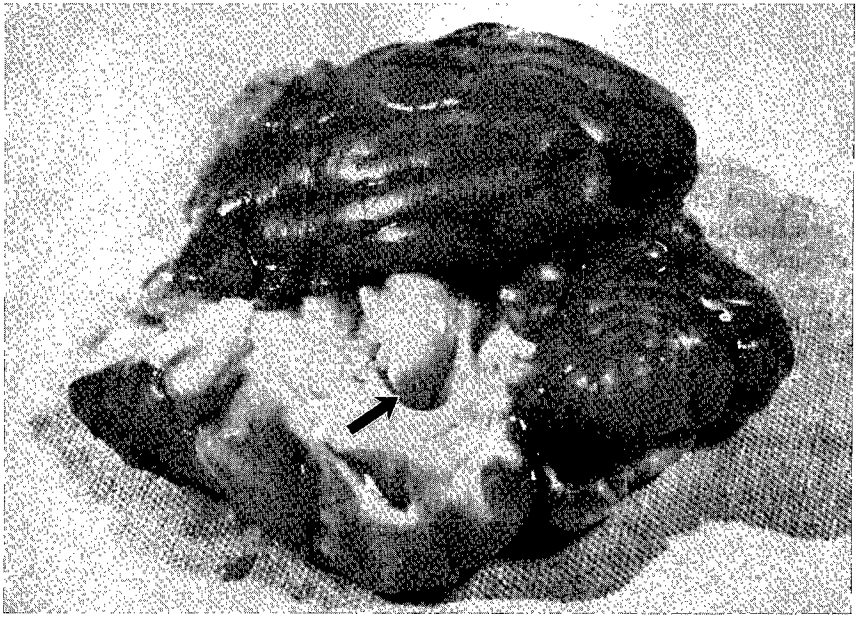
With a pair of scissors, small transverse sections (2-3 mm in thickness) of brain tissue (Ammon's horn, cerebrum, or cerebellum) are cut and placed on clean blotting-paper or a wooden tongue-depressor, cut surface facing upward (see Fig. 4 and 5). A clean microscope slide is then touched against the cut surface of the section and pressed gently downwards with just enough pressure exerted to create a slight spread of the exposed

FIG. 2. INCISION TO LATERAL VENTRICLE SEPARATED, SHOWING AMMON'S HORN *



By courtesy of US DHEW-PHS-CDC

FIG. 3. CLOSE-UP OF AMMON'S HORN * BULGING FROM FLOOR OF LATERAL VENTRICLE



By courtesy of US DHEW-PHS-CDC

* Indicated by arrow

surface of the tissue against the glass slide. According to the size of the section, three to four impressions can be made on one slide (see Fig. 6 and 7). *While still moist*, the slide is flooded with Sellers' stain (see Fig. 8), allowed to remain for a few seconds, rinsed under the tap, and dried at room temperature without blotting. The preparation is then ready for examination. The impression may be examined directly under oil, or covered with

FIG. 4. TAKING OF SPECIMEN FROM AMMON'S HORN FOR SLIDE PREPARATIONS

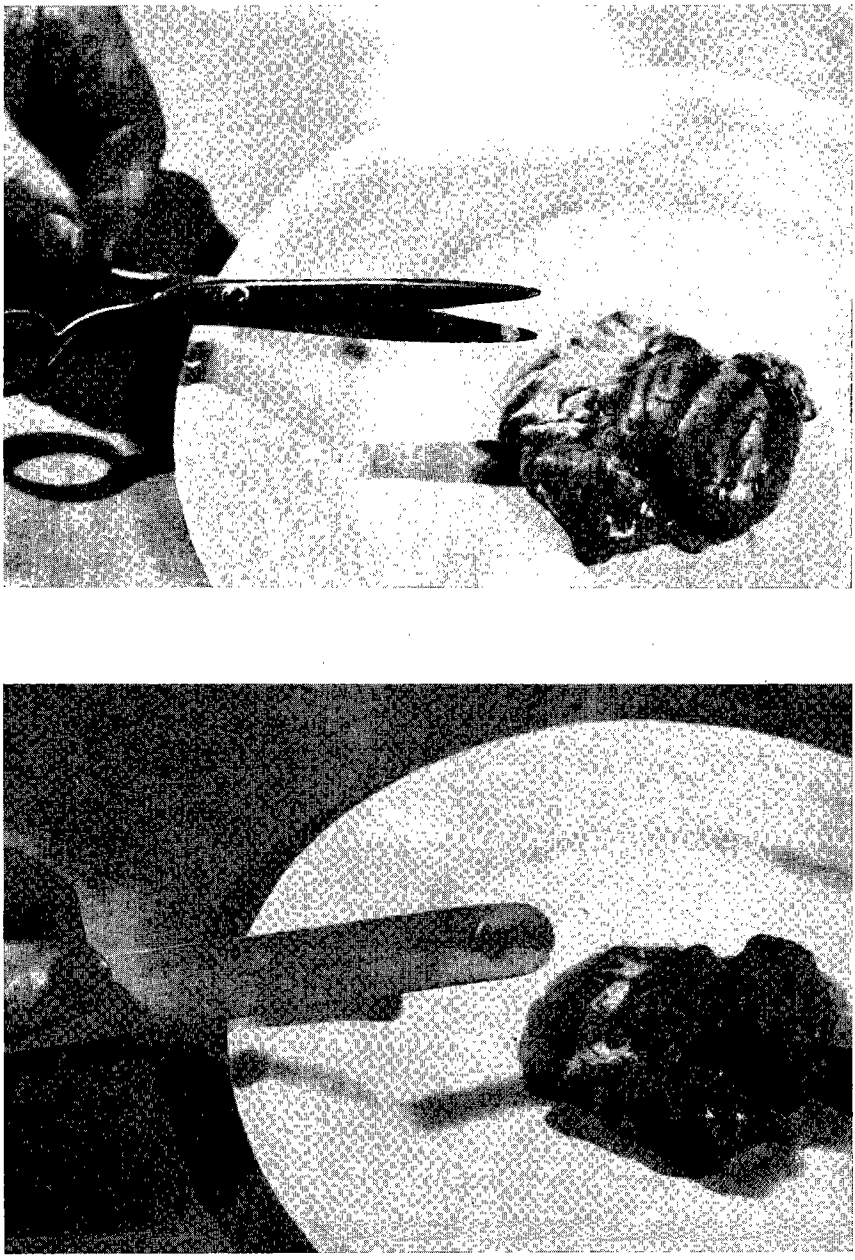
By courtesy of US DHEW-PHS-CDC

a cover-slip mounted in balsam. This method is preferred over others because a maximum amount of nerve tissue can be concentrated in a small area with a minimum amount of cellular damage.

Smear method

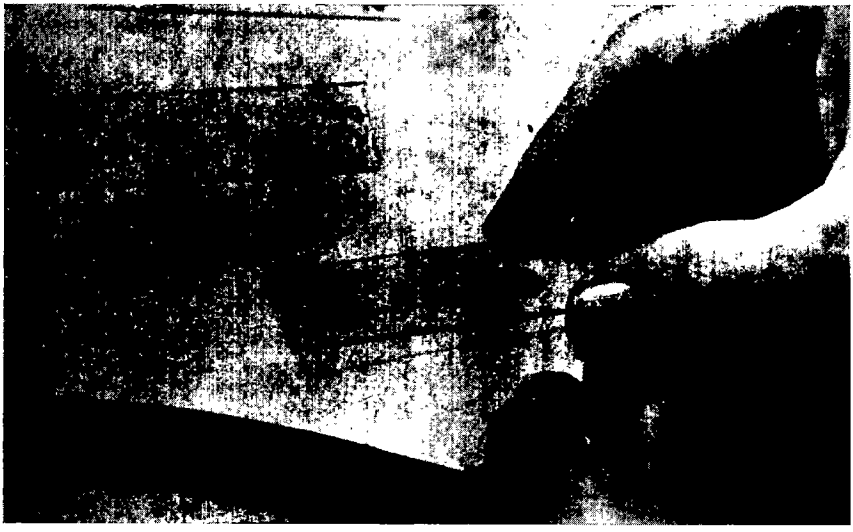
The spread-smear method consists of placing a very small section of brain tissue on one end of the slide. Another slide is used to crush the section of tissue against the first slide and is then drawn across the length

FIG. 5. TRANSFER OF SPECIMEN TO WOODEN SPATULA BEFORE MAKING IMPRESSIONS



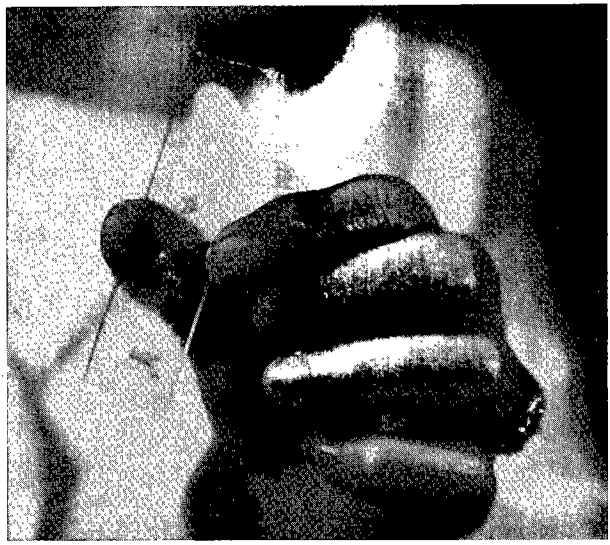
By courtesy of US DHEW-PHS-CDC

FIG. 6. IMPRESSION METHOD OF SLIDE PREPARATION



By courtesy of US DHEW-PHS-CDC

FIG. 7. CLOSE-UP OF SEVERAL IMPRESSIONS ON A SLIDE



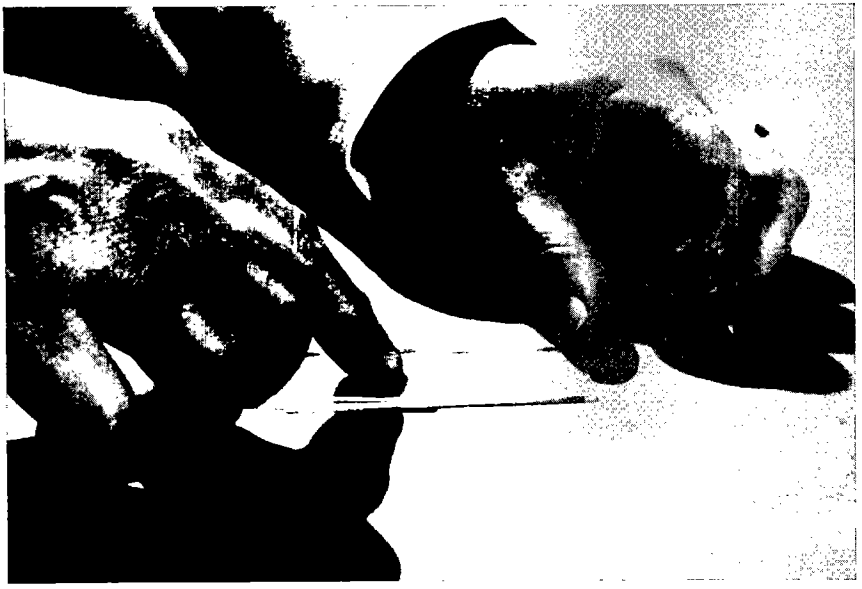
By courtesy of US DHEW-PHS-CDC

FIG. 8. IMMEDIATE IMMERSION OF SLIDE IN SELLERS' STAIN WHILE TISSUE FILM IS STILL MOIST

By courtesy of US DHEW-PHS-CDC

of the slide (see Fig. 9). The result is a fairly homogeneous spread of a thin film of film of tissue covering about three-quarters of the area of the slide.

FIG. 9. SMEAR METHOD OF SLIDE PREPARATION



By courtesy of US DHEW-PHS-CDC

In this spread-smear technique there is a copious concentration of tissue and a rather extensive area for examination. Care should be taken not to use too large a tissue-section, as this will result in an excessively thick film, making proper staining and microscopic examination impossible. The impression method, however, gives superior results.

" Rolling " method

The last method, the "rolling" technique, consists of cutting a piece of brain tissue about the size of a fresh garden-pea, and rolling or teasing it gently (cut surfaces downward) over the entire surface of the slide with a toothpick or wooden applicator.

The staining procedure of Sellers is recommended here because of its accuracy and simplicity. In this technique, no preliminary fixation is required, since the tissue film is fixed and stained simultaneously, making it one of the most rapid and easily handled methods.

Preparation of Sellers' Stain *

Examination of slide

Time may be saved in microscopic examination by a study of the stained slide under low power at first, selecting areas containing numerous large neurons to be examined for Negri bodies under immersion oil (see Fig. 10 and 11).

FIG. 10. LOW-POWER VIEW OF IMPRESSION, SHOWING FIELD (UPPER HALF) RICH IN NEURONS FOR EXAMINATION UNDER HIGH POWER

By courtesy of US DHEW-PHS-CDC

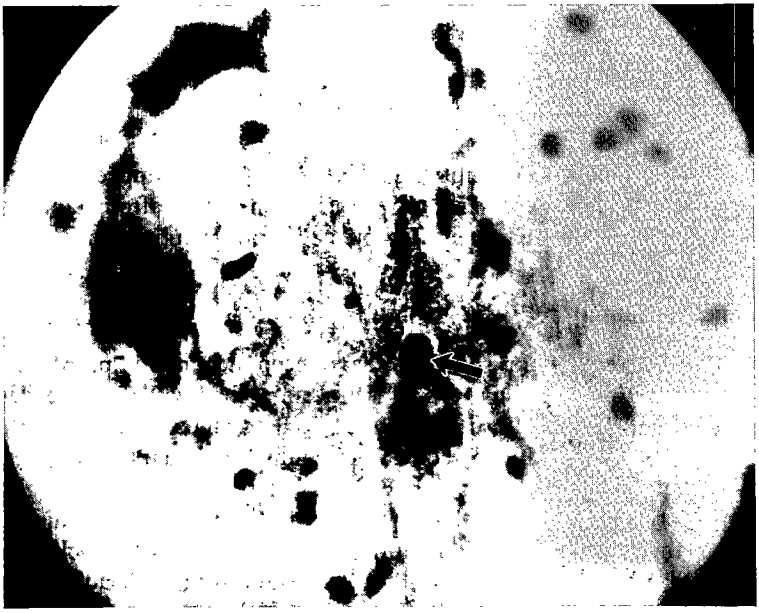
Magnification ×200

Sellers' stain shows the Negri body well-differentiated in magenta or heliotrope to bright red, with well-demonstrated dark-blue to black basophilic inner bodies. All parts of the nerve cell stain blue, and the interstitial

* This subsection was very kindly contributed by Thomas F. Sellers, Director Emeritus, Georgia Department of Public Health, Atlanta, Ga, USA.

tissue stains pink. Erythrocytes stain copper-colour (orange-tinged red) and can be easily differentiated from the magenta-tinged red of the Negri bodies (see Plate I A, facing page 48).

FIG. 11. HIGH-POWER VIEW OF NEGRI BODY*



By courtesy of US DHEW-PHS-CDC

* Indicated by arrow Magnification ×900

Stock solution

(1) Methylene blue (Colour Index[1] No. 52 015, or Schultz[2] Index No. 1038) . . . . . 10 g
Methanol (absolute acetone-free) to make. . . . . 1000 ml

(2) Basic fuchsin (Colour Index[1] No. 42 510, or Schultz No. 780) . 5 g
Methanol (absolute acetone-free) to make . . . . . 500 ml

Other suitable dyes, such as those from Gruebler & Co., Leipzig, Gurd & Co., Ltd, London, and National Aniline Co., New York, may also be used.

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[1] Society of Dyers and Colourists and the American Association of Textile Chemists and Colorists (1956) *Colour Index*, 2nd ed., Bradford.
[2] Schultz, G. (1928-1934) *Farbstofftabellen*, 7. Aufl., Leipzig.

The stock solutions are stored in screw-capped or ground-glass-stoppered bottles. Certified biological stains are preferable. The dry dyes should be selected for high dye content—methylene blue, preferably not less than 85%; basic fuchsin, preferably not less than 92%. While absolute acetone-free methanol is recommended, chemically pure (C.P.) methanol meeting American Chemical Society specifications, may be substituted if desired.

Staining solution

Methylene blue (stock solution No. 1)	2 parts
Basic fuchsin (stock solution No. 2)	1 part

Mix thoroughly but do not filter. Store in screw-capped or ground-glass-stoppered container. The mixed stain improves after standing for 24 hours, and keeps indefinitely if protected from evaporation.

Adjustment of stain

When the stock stain solutions have been accurately prepared, the above proportions will usually produce a stain that will give the desired colour differentiation. However, it is as well to make a trial stain, and if the results obtained do not equal those illustrated in Plate I A (facing page 48) the stain may readily be adjusted. If the stroma is a bright red rather than rose-pink in the thinner areas, and the overall staining effect is reddish, the fuchsin is too dominant. Add methylene-blue stock solution in measured amounts, checking with a trial stain after each addition until the desired colour balance is obtained. When the methylene blue is too dominant the Negri bodies are a deep muddy maroon colour and the nerve cells stain too deeply. Adjustment may be made with the fuchsin stock solution in this case.

If the stock solutions are protected from evaporation, more stain may be subsequently prepared using the stock solutions in the adjusted proportions.

Staining procedure

(1) Prepare smears or impressions in the usual manner (see page 27); no fixation is required.

(2) Immediately, *while the preparation is still moist*, immerse it in the stain solution for 1-5 seconds, depending on the thickness of the smear.

(3) Rinse quickly in running water, and air-dry without blotting.

In some regions tap-water is not satisfactory for rinsing purposes. The suitability of the water may be determined by comparing preparations

rinsed with tap-water and others rinsed with distilled water containing M/150 phosphate buffer, pH 7.0.

When not in use, the stain must be kept in a tightly-closed container to prevent evaporation, which tends to make the fuchsin too dominant. The addition of absolute methanol will restore the proper balance. It is convenient to keep the staining solution in a screw-capped Coplin jar for daily use. If this is not available the stain may be stored in a ground-glass-stoppered dropper bottle and the smear flooded with the stain. Staining in this manner will not be satisfactory unless the entire process can be completed within a few seconds.

The best results with the stain are obtained when the brain tissue is fresh. As decomposition sets in, the characteristic colour differentiation is affected, and although the Negri bodies retain their staining quality, the smear as a whole becomes too red, or at times too blue, and identification of the bodies becomes more difficult.

The Negri Body: Differential Diagnosis

Although generally rounded in form, the Negri body may be found to assume any shape. At various times in different laboratories it has been demonstrated to be round, oval, spheroid, amoeboid, elongate, triangular, etc. By the same token, there is great variation in size ; generally it is found within the limits of 0.24 μ and 27.0 μ. It is characteristically acidophilic in staining reaction, and takes on the pink to purplish-pink colour in differential stains which use basic fuchsin or eosin with methylene blue as their base.

The position of the Negri body within the neurone is intracytoplasmic. Classically, it is found between the nucleus and one corner of the neurone, or in the prolongation of the cell body. However, it should be stressed here that the intracytoplasmic position of the Negri body can be expected with reasonable consistency only in histological sections of the brain. In the simple tissue-application techniques described above, the histological pattern is disturbed and one may very often see well-formed Negri bodies which appear to be entirely outside the neurone. Thus, in methods such as the impression, smear, or rolling techniques, the intracellular position of the Negri body is not required as a diagnostic criterion, and Negri bodies which satisfy the requisites of morphological identification, whether inside or outside the neurone, are sufficient to establish a positive diagnosis.

The most characteristic feature of the Negri body is its internal structure. It is this feature which serves as the essential criterion for positive identification in the techniques described in this section. The matrix of the Negri body has an acidophilic staining reaction, and contained within this magenta-red structure are small inner bodies (Innerkörperchen),

basophilic granules which stain dark-blue to black. The size of these inner granules generally varies from 0.2 μ to 0.5 μ. Classically, the well-formed Negri body—the so-called textbook picture—will have its inner granules arranged in rosette fashion, with one large centrally-placed body and a series of smaller granules arranged neatly around the periphery of the Negri body. It should be pointed out, however, that this picture is the exception rather than the rule, and it is very rare indeed that such an orderly arrangement of the inner granules is seen. For purposes of diagnosis it is sufficient to establish the presence of these dark-blue-staining granules, regardless of their numbers or pattern of distribution within the matrix of the Negri body.

There is universal agreement that the Negri body is specific for rabies, and its presence always indicates this infection. Furthermore, a fully-formed Negri body cannot be confused with anything else. However, in the diagnostic laboratory other types of inclusion bodies are sometimes encountered in animal brains and, because of certain similarities, may be mistaken for Negri bodies. This is particularly true of the dog, fox, cat, and laboratory white mouse. In the brains of dogs and foxes, the acidophilic inclusion bodies of canine distemper or Rubarth's disease (canine infectious hepatitis, fox encephalitis) are occasionally encountered. These seem to occur more often in the thalamus and lentiform nuclei than in the hippocampus. By the same token, the brains of non-rabid cats and laboratory white mice occasionally contain non-specific acidophilic inclusion bodies when presented for rabies diagnosis. All these non-rabies inclusion bodies have the same staining characteristics for Sellers' stain, and they cannot be differentiated from each other with the techniques described above. However, the important thing is that these non-rabies inclusions, as a group, can be differentiated from Negri bodies with the use of Sellers' stain. The following outline may be used as a guide in this differentiation :

Negri bodies	*Non-rabies inclusion bodies*
Presence of basophilic inner granules	Absence of internal structure *
Heterogeneous matrix	Homogeneous matrix
Less refractive	More refractive
Magenta (heliotrope) tinge	Colour more acidophilic (pinker)

* See Plate I B, facing page 48

Small atypical intracytoplasmic inclusion bodies are sometimes found in animals killed during the early stages of rabies. For that reason, it is imperative to hold biting and suspect dogs in quarantine, rather than to kill them immediately and send the brain to a laboratory for diagnosis (see page 17). There is a double reason for this. First, it will allow observation for symptoms of rabies which may make possible a clinical diagnosis

of the disease. Secondly, the longer the animal is allowed to live, the better the chance of obtaining a positive microscopic diagnosis. The length of clinical illness in rabies is directly related to the presence, size, abundance, and development of Negri bodies. Thus, if the disease runs its full course, Negri bodies which are larger, more abundant, and fully formed with good internal structure, are more likely to be found.

Biological Diagnostic Test: Preparation for Mouse Inoculation

Since Negri bodies cannot always be found in the brains of animals dying of rabies, it is important that animal inoculation for demonstration of the virus be done on Negri-negative specimens. Extensive surveys of large numbers of rabies cases have shown that 10-15% of cases proved positive by mouse inoculation had been missed by direct-smear microscopic examination for Negri bodies. It is therefore strongly recommended that laboratories that furnish rabies diagnostic services be equipped to carry out animal inoculation tests on Negri-negative brain tissues.

In the past, the guinea-pig and rabbit have been considered the most suitable test animals for this purpose. Since the demonstration that the intracerebral injection of rabies virus into white mice produces typical and constant infection, the white mouse has become the test animal of preference. The chief advantages of the mouse are the low cost, making it possible to use several animals for one specimen, the relatively short incubation period for street virus, and the consistency of production of Negri bodies in the brains of mice inoculated intracerebrally with street virus.

A positive microscopic diagnosis is sufficient proof of the presence of rabies. When the microscopic examination proves Negri-negative or questionable, no time should be lost in taking samples of the cerebral cortex, cerebellum, and Ammon's horn on each side of the brain, plus a sample from the medulla-brain-stem. These should be pooled in the emulsifier in preparation for the mouse inoculation test described in chapter 6, page 69. It is quite important to achieve complete representative sampling of all those parts mentioned on each side for pooling, since there is often great variability in the virus distribution through the brain.

Antibacterial Agents for Contaminated and Decomposed Specimens

It is often difficult to obtain from the field animal brains that are bacteriologically sterile. The head may have been in transit for a long time, or the animal may have been picked up long after death, or shot, or clubbed on the head. Also, the cause of death may have been a bacterial encephalitis.

Intracerebral injection of bacteria may cause the death of inoculated mice in one, two, three, or more days, before any rabies virus that may be present in the inoculum has had its full incubation period. On the other hand, inoculated mice may live long enough for rabies incubation to be complete, may pass through the typical rabies symptoms of tremors, paralysis, and prostration, followed by death, and may show typical Negri bodies and many bacteria in their brain smears.

When bacterial contamination is suspected—for example, if the animal brain is decomposed or when many bacteria are demonstrated on the original brain smears—it is best to treat the brain suspension with an antibacterial agent before inoculating it into mice. Of the following agents, penicillin and streptomycin [1] will give the best results. If these antibiotics are not available, any of the other agents may be used.

(1) *Glycerol.* Place the brain specimen in pure glycerol for 48 hours.

(2) *0.5% phenol.* Make up 0.5% phenol in physiological salt solution. This is used as the diluent for making up the 10 % tissue inoculum. Hold for 6 hours or overnight. If held for 6 hours, keep the suspension at room temperature. If held for over 6 hours, keep the suspension overnight in the refrigerator.

(3) *1 : 5000 thiomersal (merthiolate).* Make up a 1 : 5000 solution of thiomersal in physiological salt solution. This is used as the diluent for making up the 10% tissue inoculum. Hold for 6 hours or overnight. If held for 6 hours, keep the suspension at room temperature. If held for over 6 hours, keep the suspension overnight in the refrigerator.

(4) *10% ether.* Place one part of ether in ten parts of tissue suspension ; shake in a stoppered bottle and leave at room temperature for two hours ; use a sterile cotton plug to allow for evaporation of ether.

Caution : If all the ether has not evaporated, its intracerebral injection will cause the almost instant death of the mice.

(5) *Penicillin and streptomycin.* Add 500 units of soluble sodium penicillin G and 2 mg of streptomycin per ml of tissue suspension. Allow to stand for 30 minutes at room temperature before injection. This amount is usually enough, but for very heavily contaminated brains or salivary glands, as many as 1000 units of penicillin and 3 mg of streptomycin may be used.

In order to help detect the possible presence of contaminating bacteria, a portion of all tissue emulsions should be cultured in dextrose infusion broth or similar media, and streaked on a blood-agar plate. The recommended amount is about 0.1 ml of emulsion in 3 ml of broth. Incubate overnight at 37.5°C.

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[1] Johnson, H. N. (1942) *Illinois med. J.*, **81**, 382.

Early deaths (1-3 days) among the infected mice may be attributed to the presence of contaminating bacteria if the cultures show moderate to heavy growth, and if many bacteria are found in the brain smears of the dead mice.

HISTOPATHOLOGICAL DIAGNOSIS

PIERRE LÉPINE [1]

In principle, the histopathological diagnosis of rabies consists in recognition of the presence in the animal of acute encephalomyelitis which can be ascribed to a specific agent, namely the rabies virus.

If the animal has died from rabies, it is normally easy to detect the specific lesions ; if the animal has been killed, death may have occurred before the appearance of specific lesions (Negri bodies). Consequently, any animal in which the cerebrospinal axis (brain, medulla, ganglia) shows the slightest sign of lesions, particularly infiltrations, should be regarded as suspect, no matter how small the lesions may be.

After correct removal, the brain should be carefully dissected. Smears or impressions should first be examined for Negri bodies (see chapter 3, page 26). If any are found, this establishes the diagnosis. If this examination is negative, rabies is not thereby eliminated. A regular histopathological examination should be made of sections stained after embedding by a rapid method. At least six samples should be examined, corresponding respectively to Ammon's horn (both sides), the cerebral cortex (motor area), the cerebellum, the medulla, and a ganglion (gasserian or upper cervical).

The sections should be examined for :

(*a*) Signs of meningo-encephalomyelitis, i.e., meningitis, meningeal infiltration, perivascular cuffing, parenchymatous infiltration, formation of encephalitic nodules (Babès' nodules), and ganglion infiltration with satellitosis and neuronophagia (lesions of van Gehuchten and Nélis) (see Fig. 1 and 2).

These lesions may be detected by any staining method (e.g., haematoxylin-eosin, polychrome methylene blue). They show the existence of encephalomyelitis and enable a *tentative* diagnosis of rabies to be reached.

(*b*) Specific lesions. The different types of neurones should be examined for Negri bodies and lesions of fixed-virus rabies.

The Negri bodies are found especially in the central pyramidal layer of Ammon's horn (see Fig. 3) and the hippocampus, in the lower loop and the middle layer of the ganglioneurones of Ammon's horn and, less frequently,

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[1] Head, Virus Division, Pasteur Institute, Paris, France.

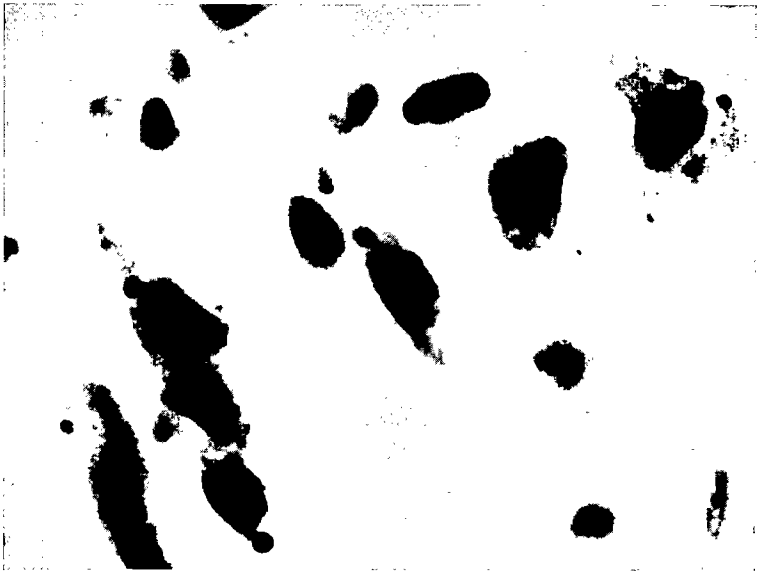
FIG. 1. NORMAL GASSERIAN GANGLION

Ganglioneurones with a single layer of satellite cells

FIG. 2. RABIES : INFILTRATED GASSERIAN GANGLION

Infiltration, satellitosis, and neuronophagia (lesion of van Gehuchten & Nélis)

FIG. 3. RABBIT : STREET RABIES



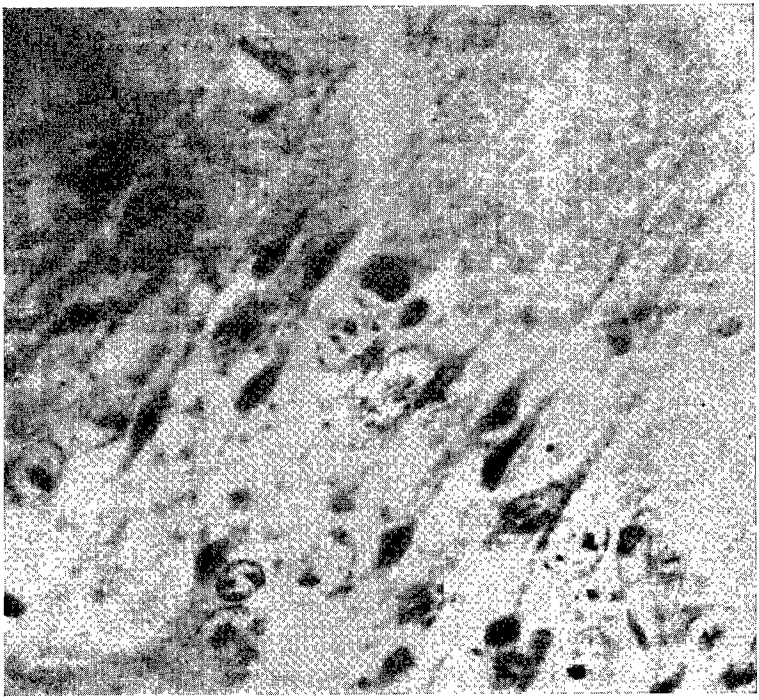
Mann stain

Magnification ×2000

Negri bodies in neurones of the central pyramidal layer of Ammon's horn (Staining was performed on a dog's brain that had already undergone partial autolysis.)

in the neurones of the cerebellum, the motor area of the cerebral cortex, and the medullary nuclei. They may be present in very large numbers in the ganglia but are generally small in size.

FIG. 4. RABBIT : FIXED-VIRUS RABIES



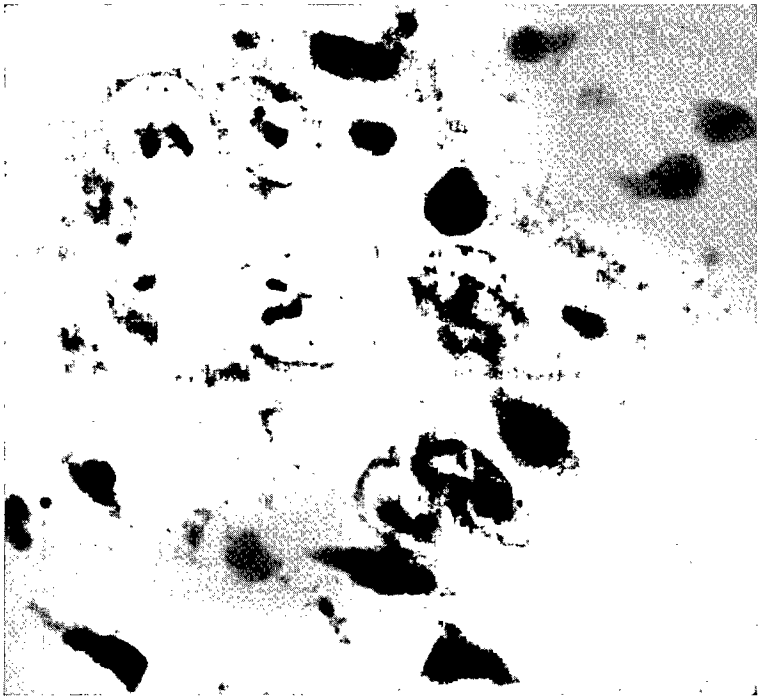
Lépine (fuchsin-safranine-blue) stain Magnification ×770

Polychromatic degeneration of neurones in outer layer of Ammon's horn

The lesions of fixed-virus rabies are found exclusively in the middle zone of the external layer of the cells of Ammon's horn (see Fig. 4 and 5). They always co-exist in a varying proportion with the lesions of street-virus rabies.

These lesions can be detected only by special staining methods (Mann, Giemsa (see Plate ID, facing page 48), Sellers, etc.). Their presence enables a *definite* diagnosis of rabies to the made.

FIG. 5. RABBIT : FIXED-VIRUS RABIES



Lépine (fuchsin-safranine-blue) stain Magnification ×1850

Detail of affected neurones : pyknosis of cellular bodies, with large quantities of polychromatic nuclear debris

BRAIN REMOVAL AND PREPARATION OF MATERIAL

Large Animals (Dogs, Cattle)

The animal is firmly secured to the autopsy table or, better, the head is separated from the body and strapped to a wooden block hollowed out for the purpose. The operator and his assistants should wear thick rubber gloves to protect their hands. Make an incision on the midline of the skull through the skin, push aside the flaps of skin, and reflect the muscles and fascia as far as the base of the skull, proceeding from the crown to a horizontal line passing through the eyes. Saw transversely through the skull at eye level and make a symmetrically placed saw-cut through the

occipital bone, followed by two more cuts, one on each side, along the temporal bone in prolongation of the cuts already made. Join up the saw-cuts by means of bone-cutting forceps, and lift up and push backwards the top of the skull. In the case of very large animals (large dogs, cows, etc.) a different method is preferable : make a longitudinal saw-cut on each side of the midline at about 1.5 cm from it, and join up these cuts by one or two transverse saw-cuts above the orbits and at the occiput so that the calvaria can be removed in two symmetrical pieces.

Once the calvaria has been removed, use fresh instruments to open the meninges, making use of serrated dissection forceps and a pair of fine, sterile scissors. The operation is performed by making an incision in the meninges, starting from the median region, along and on each side of the longitudinal sinus ; next, a second incision is made perpendicular to the first and the meningeal flaps pushed upward and backwards. Again change instruments ; cut through the medulla with a scalpel as low as possible and lift up the brain, proceeding from back to front and successively severing the pairs of cranial nerves. At the end of the operation the brain is toppled over forwards into a large sterile Petri dish where it rests on its upper surface. In hot weather, or if the brain is soft (cadaveric brain), it should be cooled to +5°C (refrigerator) to give it a firmer consistency before dissection.

Examination of the brain

Note whether or not there is congestion of the cerebral vessels or exudate in the meninges, etc. Dissect the brain as follows :

(1) With a brain knife separate the two hemispheres longitudinally, after having detached the cerebellum and the medulla.

(2) Look for the hippocampus and Ammon's horn. This may be done in two ways :

(*a*) Cut across the brain transversely, starting from the base behind the optic chiasm and proceeding towards the lower third of the convexity. The third ventricle appears on the cut surface ; Ammon's horn is seen as a whitish fold resembling a large bean cut transversely, and can easily be removed.

(*b*) Alternatively, a longitudinal incision may be made externally in the posterior third of each cerebral hemisphere about 1.5 cm from the midline ; the incision is continued through the grey matter and the white matter until a narrow groove, the third ventricle, is reached. The hippocampus will be seen on the floor of the ventricle in the form of a glistening white, semi-cyclindrical bulge, extending laterally on each side (see chapter 3, Fig. 3, page 28).

Cut transverse sections 1-2 mm in thickness from each hippocampus. Take similar samples from the cerebral cortex (motor area), the cerebellum, and the medulla.

When using the impression method, at least six slides (two for each hippocampus, one for the cerebral cortex, and one for the cerebellum) should be carefully inspected before deciding that the results are negative. If the results are negative, however, histological examinations are carried out.

Preparation of the tissue samples for histological examination

If the tissue is soft and difficult to section, prepare pieces of filter paper slightly larger than the tissue sample to be collected. Apply the piece of filter paper to the cut brain surface, grasp the edge of the filter paper with fine forceps held in the left hand, and with the right hand make a cut with e scalpel parallel to the filter paper and 2 mm or 3 mm from it, so as to ramove the piece of brain, which is immediately submerged in the fixing agent together with the fragment of filter paper to which it is adhering.

Removal of material for inoculation

In operating, care should be taken to put on one side fragments aseptically removed from the same areas (cortex, hippocampus, cerebellum, medulla) for use in animal inoculation (see chapter 6, page 69). If the brain is received in good condition and can be assumed to be sterile, the fragments are removed before any examination is made. When the brain is infected, the fragments may be removed at any time and antibiotics added, as described in chapter 3, page 40.

Small Animals (Rabbit, Guinea-pig, Hamster, Mouse)

Rabbit

The animal, resting on its ventral surface, is attached to the autopsy tray, with the head at the edge of the tray. Using a serrated dissection forceps, the scalpel and the scissors, completely scalp the head from the nape to the muzzle, removing the ears and the upper eyelids. Moisten the exposed surface of the head with iodized ethanol and rapidly flame it with a bunsen burner. Holding the muzzle of the animal with a Farabeuf forceps in the left hand, open the brain pan with three cuts of the bone forceps. Make the first two in the front part of the head, from each orbit to the midline with an upward and outward movement of eversion, opening the brain pan (parietal and temporal bones) in two flaps, to the right and to the left. The third cut is made at the occiput, with a backward movement

DESCRIPTION OF PLATE I

A. Sellers' stain showing three large Negri bodies with " Innerkörperchen "; the small round cells stained red are erythrocytes. (Magnification ×900)

B. Fox: non-specific inclusion body in cytoplasm of degenerated neurone (anterior horn cell). Note homogeneity and lack of inner structure of inclusion body. (Mann stain: magnification ×1440)

C. Street rabies—I. The cell right of centre shows the difference between the nucleolus (stained violet-red inside the nucleus) and the Negri body (in the same cell just below the nucleus). Other Negri bodies can be seen in the cytoplasm of the cell at the 10 o'clock position. (Mann stain: magnification ×680)

D. Street rabies—II. Note Negri body in cytoplasm of neurones below centre of figure. (Giemsa stain: magnification ×680)

E. Street rabies: typical Negri bodies, several showing internal structure; many micro-bodies present; several, situated at the prolongations of the neurones, appear to be extracellular. (Lépine fuchsin-safranine-blue stain: magnification ×680)

F. Fixed-virus rabies—I. Typical oxyphilic nuclear degeneration characterizing fixed-virus lesions. (Mann stain: magnification ×680)

G. Fixed-virus rabies—II. Similar type of oxyphilic nuclear lesion in a neurone in the outer layer of Ammon's horn. (Giemsa stain: magnification ×680)

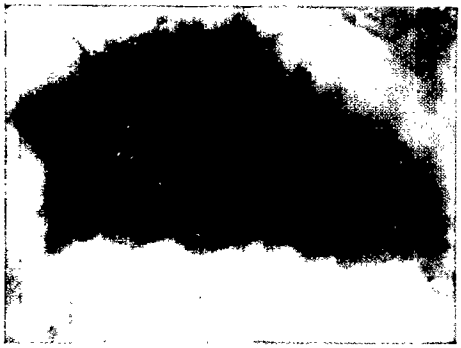
H. Onset of nuclear degeneration resulting in fixed-virus rabies lesion: small polychrome bodies fill the nucleus of the affected neurone. (Mann stain: magnification ×680)

* * *

A. is reproduced from Rivers, T. M., ed. (1952) *Viral and rickettsial infections of man*, 2nd ed., Philadelphia & London, by courtesy of J. B. Lippincott Company; B. is reproduced by courtesy of Lederle Laboratories, Division of American Cyanamid Company, Pearl River, N.Y., USA; illustrations C-H are reproduced from transparencies kindly supplied by Professor P. Lépine, Pasteur Institute, Paris, France.

PLATE I

A.

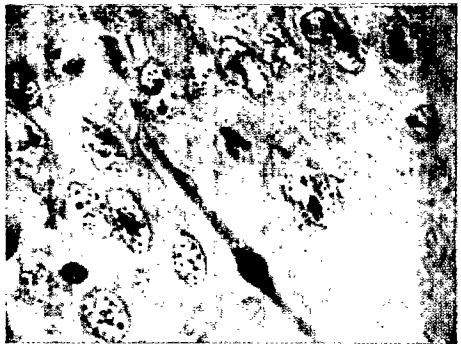


B.

C.

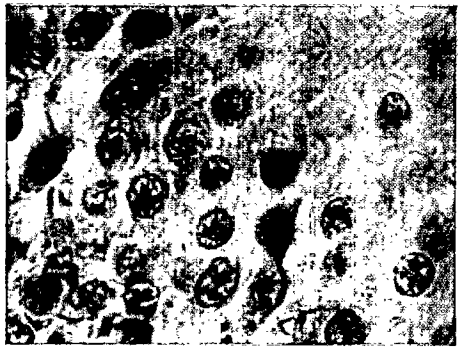
D.

E.



F.

G.



H.

of eversion which completes the clearance of the field of operation. The Farabeuf and bone forceps are then laid aside. With the fine forceps and the scissors the meninges are freed and the anterior part of the brain sectioned at the olfactory lobe ; after severing the medulla behind the cerebellum, the brain is raised in order to cut the optic chiasm and is then placed in a Petri dish where it is dissected.

With the brain lying on its dorsal surface in the Petri dish, the ventral surface facing upwards, cut the cerebral trunk at the peduncle ; then cut through the brain substance along a transverse slanting plane, starting from the optic chiasm and going towards the convexity, parallel to the posterior surface of the hemispheres and the cut surface of the cerebral trunk. A second cut made in the same way, parallel to the first and from 5 mm to 8 mm behind it, gives a transverse section of the brain which includes the hippocampal gyrus and Ammon's horn as well as the basal optic ganglion—areas of choice for the detection of Negri bodies—in addition to the cortical motor area (see Fig. 6 and 7, and Plate IE, facing page 48). A transverse section of the cerebellum makes it possible to examine Purkinje's cells and the peduncular region. Finally, a slice cut from the end of the cerebral trunk gives a section of the medulla. To reach the gasserian ganglion, cut through the petrosal bone with the bone forceps at its insertion into the sella turcica ; force apart the cut surfaces by everting the temporal bone downward and outwards. The gasserian ganglion is easily recognized from its whitish nodular appearance and its almost fibrous consistency. The ganglion is carefully removed, freed from fragments of bone, placed on a piece of filter paper, and immersed in the fixing agent with the other samples.

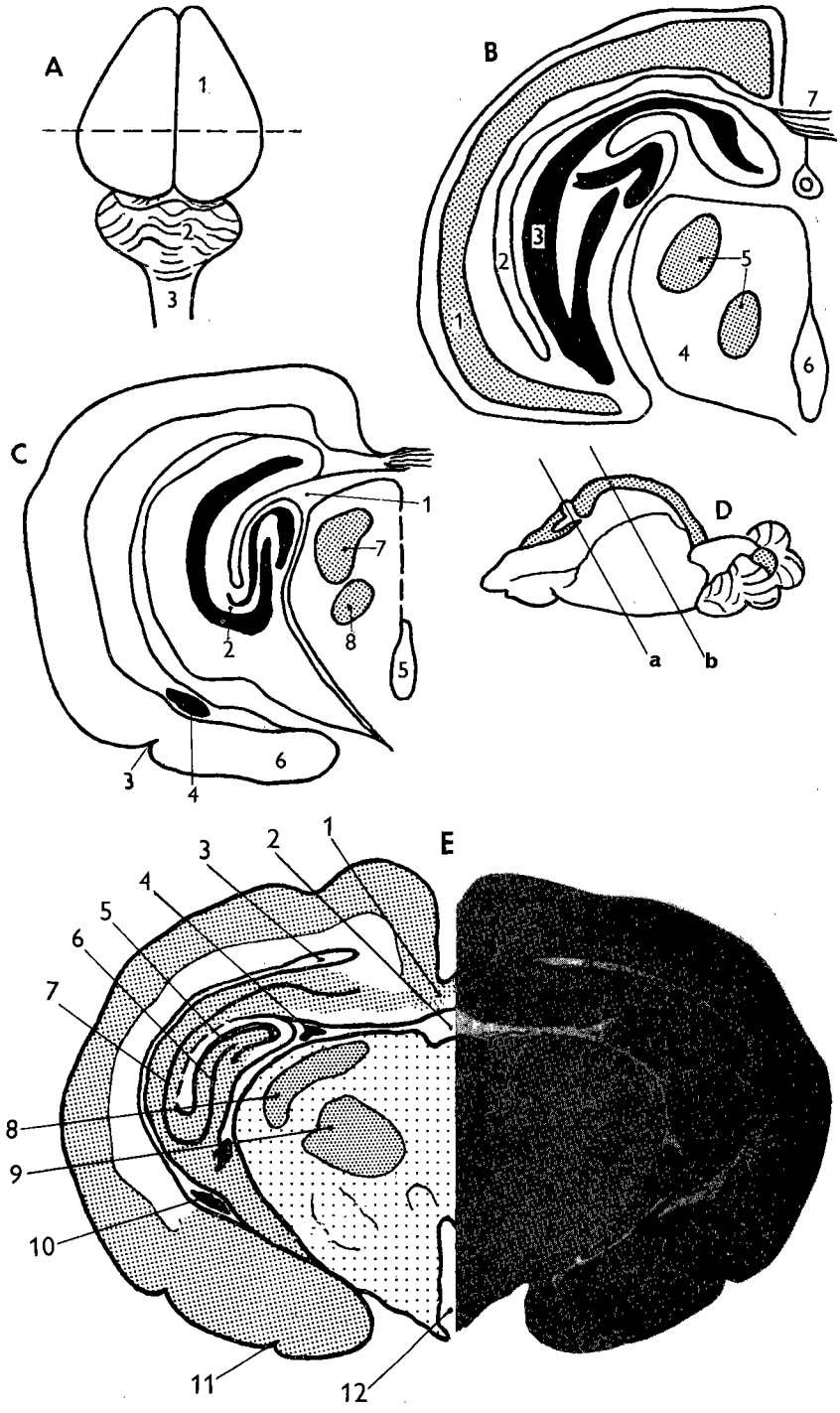
Guinea-pig, hamster

The animal is secured to the autopsy tray and the head thoroughly freed from skin. The skull is rapidly flamed. With the second pair of large sterile scissors, open the skull by means of four incisions encircling the skull cavity, the first one joining the two orbits and the other three made successively at the sides and the occipital bone. Remove the brain with the fine forceps and scissors and dissect it as described above for the rabbit. The gasserian ganglion is more difficult to locate than in the rabbit, but, with care, it can be found.

Mouse

The mouse is pinned, ventral surface downwards, on a sheet of cork, the limbs being spread out and fixed. First of all the four paws are fixed, the base of the tail is secured with a fifth pin, and a sixth is passed through the anterior extremity of the muzzle which is pulled well forward. Remove

FIG. 6. SECTIONS OF RABBIT AND MOUSE BRAIN



For explanation of this figure, see page 51.

the skin from the head. Treat the skull with iodized ethanol and flame it very gently with the pilot flame of the bunsen burner. Open the skull with the fine scissors, commencing by joining the orbits, then cutting laterally through the skull fairly low, and finally pushing back the flap thus obtained. Sever the medulla and the chiasm. Remove the whole of the brain and place it in a Petri dish. Separate the cerebellum. Cut through the brain transversely at the optic chiasm and along a plane parallel to the posterior surface of the brain, so as to obtain a cross section which is placed in the fixing agent, the anterior portion being reserved for smears and inoculations. Fix also a section of the cerebellum.

If it is wished to examine a ganglion, the upper part of the spinal cord should be detached and removed together with the upper cervical ganglion.

EMBEDDING, STAINING, AND EXAMINATION FOR NEGRI BODIES

Rapid Mercury Sublimate Fixing Agent (Lépine & Sautter, 1936)

The following mixture is recommended for rapid fixation of the nervous system : equal volumes of glacial acetic acid, acetone, and saturated solution of mercuric chloride ($HgCl_2$) in absolute ethanol.

The mixture is made in advance and keeps perfectly well. Prepare the saturated solution of mercuric chloride in absolute ethanol in hermetically sealed bottles ; hasten saturation by keeping in the incubator at 37°C. The sections should be treated with Lugol's solution or iodized ethanol before staining, in order to remove the sublimate. Mixtures containing other components, particularly formaldehyde, give distinctly inferior results, as do mixtures to which water or hydrated ethanol has been added in order to slow down fixation. Tissues other than those of the nervous system are hardened by this fixing agent.

Key to Fig. 6 (page 50)

A : Brain (mouse, rabbit) : 1. cerebrum ; 2. cerebellum ; 3. medulla oblongata

B : Section of normal mouse brain : 1. cerebral cortex ; 2. lateral ventricle (choroid plexus) ; 3. Ammon's horn ; 4. mesencephalon ; 5. inner nuclei of grey matter ; 6. median ventricle ; 7. corpus callosum

C : Section of normal rabbit brain : 1. third ventricle ; 2. Ammon's horn (middle layer) ; 3. rhinal fissure ; 4. choroid plexus ; 5. infundibulum ; 6. hippocampal gyrus ; 7. lateral geniculate ganglion ; 8. ventral nucleus of thalamus

D : Rabbit brain after removal from cranial cavity : inverted, lateral aspect. Left—anterior portion ; centre—temporal and parietal lobes ; right—cerebellum. Section is performed anterior to *a* (through optic chiasm) and posterior to *b* ; the portion *a-b* is immersed in fixative

E : Rabbit brain : transverse section *a-b* : 1. corpus callosum ; 2. third ventricle ; 3. lateral ventricle ; 4. choroid plexus ; 5. Ammon's horn (middle layer) ; 6. Ammon's horn (inner layer) ; 7. Ammon's horn (outer layer) ; 8. lateral geniculate ganglion ; 9. ventral nucleus of thalamus ; 10. lateral choroid plexus ; 11. rhinal fissure ; 12. infundibulum

Rapid Fixation with Susa's Mixture

Rapid fixation can also be effected with Susa's mixture. For this purpose, the formula given by Langeron (1949) is recommended. First prepare a stock solution of the following composition :

sodium chloride	5 g
mercuric chloride ($HgCl_2$)	45 g
distilled water	800 ml

This mixture is stable and keeps indefinitely.
Immediately before use, prepare the following mixture :

stock solution	80 ml
acetic acid	4 ml
40% formaldehyde solution	20 ml
trichloracetic acid	2 g

Cut the tissue in thin slices and fix for a few hours in this mixture. The fixation time should not exceed 48 hours. When fixation is complete, do not wash the tissue with water but treat it directly with iodized ethanol and then with 96% ethanol and absolute ethanol prior to embedding.

Fixation with Bouin-Dubosq-Brazil Mixture

When ultra-rapid fixation is not necessary and 24 hours are available for the purpose, the best fixing agent is beyond doubt Bouin-Dubosq-Brazil mixture. This can be used for all examinations not requiring a special fixing agent and is particularly suitable for the staining of nuclear and cytoplasmic inclusions. The formula is as follows :

commercial 40% formaldehyde solution	500 ml
96% ethanol	1100 ml
distilled water	100 ml
glacial acetic acid	120 ml
picric acid	8 g

Fixation is followed, without washing, by dehydration in 96 % ethanol and then in absolute ethanol. The specimens are embedded in paraffin, using the customary technique.

Rapid Method for Histological Embbeding

For slices of brain tissue 1 mm in thickness, fixation is complete in 15-30 minutes if a rapid fixation method is employed. The tissue is transferred directly to absolute ethanol, with which it is treated for 20-30 minutes in two baths, followed by two changes of toluene and two of paraffin, each lasting 15 minutes. Including the time necessary for cutting and staining the sections, the preparation is ready for microscopic examination from three-and-a-half to four hours after autopsy of the animal.

Embedding Using Dioxan

It is sometimes advantageous to use a solvent for paraffin which is also miscible with water. The number of manipulations is reduced and for fairly thin tissue fragments there is a gain of time. The dioxan technique is suitable for this purpose.

Fix the tissue in thin slices, not exceeding 5 mm in thickness. In principle, any fixing agent can be used. It is preferable, however, to employ Bouin's dioxan mixture as given by Lison (1936) :

dioxan saturated with picric acid	8.5 volumes
commercial 40% formaldehyde solution . .	1 volume
glacial acetic acid	0.5 volume

Next embed without washing, following the procedure given below (Mossman, 1937) :

dioxan I	1 hour
dioxan II	1 hour
dioxan III	2 hours

followed by :

paraffin I	15 minutes
paraffin II	45 minutes
paraffin III	2 hours

Use fresh paraffin for embedding.

Take care to keep the dioxan in well-stoppered bottles (risk of chronic poisoning accompanied by anaemia caused by the vapour if the dioxan is kept in open bottles), and ensure that it remains anhydrous by adding pieces of quicklime (CaO).

Staining by Sellers' Method

Sellers' method may be used either in combination with rapid dioxan embedding (as described by Schleifstein, 1937) or for staining sections prepared in the normal way, adopting the technique of Nobel & Marton which ensures rapid and well-differentiated staining. Thus, the simplicity of Sellers' method of staining is combined with the greater accuracy afforded by histological examination, so that the Negri bodies can be observed *in situ* in the cells, and all possibility of error is excluded.

The preparation of Sellers' stain is described in section 3, page 34. After being freed from paraffin, the sections are stained by immersion in a mixture of 6 ml of basic fuchsin stock solution, 20 ml of methylene blue stock solution and 50 ml of absolute methanol.

The time required for staining depends on the thickness of the section ; it is usually between 2 minutes and 10 minutes. The stained sections are

washed in tap water, dried with filter paper without washing with alcohol, and mounted in balsam. The Negri bodies stain deep magenta red and appear as vacuoles in the blue-violet of the neurone. The internal structures of the Negri bodies are stained deep blue, as are the nucleoli of the nerve cells. The erythrocytes stain copper red, and it is impossible to confuse them with Negri bodies.

Staining of Negri bodies by Mann's Method

This classical method gives sections which are permanently stained, with very fine differentiation of the Negri bodies. It is an excellent demonstration method, but it requires time and a certain knack for full success.

Prepare the following mixture immediately before use :

methyl blue (not methylene blue), 1% aqueous solution .	18 ml
1% aqueous eosin solution	23 ml
distilled water	49 ml

Stain for 24 hours at laboratory temperature, or for 6-14 hours at 38°C (in this case first treat the section with an ethanol-formaldehyde mixture to render the gelatine insoluble, since otherwise the sections come loose).

Wash, first with tap-water, and then rapidly with absolute ethanol.

For differentiation use the following alcoholic caustic soda solution :

1.5% solution of caustic soda in ethanol . .	1 ml
absolute ethanol	30 ml

Leave the section in this solution until it is stained pink (about 10 minutes). As soon as this stage is reached, wash the preparation well with tap-water. The section should take on a sky-blue colour ; if not, treat it with water containing acetic acid (2 drops of acetic acid in 40 ml of distilled water) for 1 minute.

Dehydrate rapidly (absolute ethanol) treat with xylene, and mount in balsam.

Result : Negri bodies, vermilion red ; nucleoli of the neurones, violet red ; chromatin, blue ; cells, dark blue ; stroma, pale blue ; erythrocytes, pink (see Plate I, C, F & H, facing page 48).

On substituting phloxin B for eosin in the same proportion, the preparations obtained are less attractive (purplish-blue or mauve background instead of sky-blue) but the inclusions (Negri bodies) are more numerous and more striking.

Staining of the Negri Bodies by the Fuchsin-Safranine-Blue Method (Lépine, 1935)

After being well fixed, the tissue is embedded in paraffin, cut into thin sections, and freed from the paraffin. Stain for 10 minutes with the following mixture :

(1) basic fuchsin . . . . . 1 g
50% ethanol . . . . . 200 ml
(2) 0.2% aqueous solution of safranine.

Mix equal parts of (1) and (2) and store in a dropping bottle ; the mixture is fairly stable and keeps for some time.

Discard the stain, cover the section with a mixture of ethanol and acetone (equal parts) to remove excess stain, and wash rapidly : the section is coloured red. Stain for from 15 seconds to one minute with Unna's polychrome methylene blue (10% dilution) or with permanganate blue prepared by Stévenel's method (1918) and used undiluted. Discard the stain : the section is deep violet in colour. Differentiate in ethanol-acetone for a few seconds : the section becomes blue. Wash the preparation immediately in running water to remove the excess of blue stain, and again treat with ethanol-acetone. Then, without washing, partially dehydrate by shaking in a Borrel tube filled with absolute ethanol. The remaining stain is removed from the section which becomes differentiated, taking on a pink-lilac tint varying in paleness according to the thickness of the section. Rapidly complete dehydration in absolute ethanol, carefully remove the alcohol in several changes of xylene or toluene, and mount in balsam.

Result : stroma, very pale pink with nerve fibres a deeper pink ; neuroglia and leukocytes, purplish-blue ; neurones, light blue ; chromatin, deep purple with the nucleolus a vivid red ; pathological formations particularly well shown : nuclear inclusions and oxyphilic substances, bright pink ; Negri bodies, poppy-red to mauve pink, with the internal structure lilac (see Plate I, E & G, facing page 48).

Method of Stovall & Black

A popular staining method used in the USA is that described by Stovall & Black (1940) using acetone fixation and sequence staining. The following description is taken from Lillie (1948).

1. Stain for 2 minutes in a 1% alcoholic solution of ethyl eosin (Colour Index [1] No. 45 386) adjusted to pH 3.0 with N/10 hydrochloric acid.
2. Rinse in water.

[1] Society of Dyers and Colourists and the American Association of Textile Chemists and Colorists (1956) *Colour Index*, 2nd ed., Bradford.

3. Stain for 30 seconds in 0.23% methylene blue in 22% alcohol buffered to pH 5.5 with acetic acid/sodium acetate buffer.
4. Differentiate in 0.38% acetic acid (13 drops in 60 ml of water) until sections are brownish red.
5. Wash, dehydrate, and clarify (xylene or toluene).
6. Mount in balsam.

Results : Negri bodies are brownish to pure red, nucleoli pale blue, other structures pink.

Lillie also recommends the following modification of the above method (Mossman, 1937), which has given good results :

1. To 90 ml of absolute ethanol or 94 ml of 95.5% ethanol add 3.25 ml of 1% acetic acid and make up to 100 ml with water ; dissolve 950 mg of ethyl eosin in this mixture. Stain for 2 minutes in this solution.
2. Wash in alcohol and in water.
3. Counterstain in 0.5% methylene blue if the section was fixed in formaldehyde, or in alum haematoxylin if the section was fixed in ethanol.
4. Differentiate in 0.25% acetic acid for 2 to 5 minutes.
5. Wash, dehydrate, and clarify.
6. Mount in balsam.

FIG. 7. STREET RABIES : AMMON'S HORN (MIDDLE LAYER)

Mann stain Magnification ×1100

Appearance under white light ; many Negri bodies in the neurones

Detection of Negri Bodies with the Fluorescence Microscope
(Levaditi et al., 1948)

This method makes use of the fluorescence of the Negri bodies when they have been impregnated with a fluorochrome and excited by exposure to light of a wavelength of 4000 Å.

(1) Fix the brain tissue and embed in paraffin as usual. Make fine sections, mount on slides, and treat successively with xylene, absolute ethanol, and water to remove the paraffin.

(2) Next, stain by immersion for 30 minutes in an aqueous 0.2% solution of thioflavine S. Without washing the preparation, treat it for a few seconds with absolute ethanol and then with toluene. Mount in balsam between the slide and the cover-slip, applying as thin a layer of balsam as possible. It should first be ascertained that the balsam does not contain substances that fluoresce in ultraviolet light.

(3) Next examine with the fluorescence microscope, using a filter to block wavelengths longer than 5150 Å. Locate Ammon's horn under

FIG. 8. STREET RABIES : AREA OF BRAIN, TISSUE BLOCK, AND CELLS IDENTICAL WITH THOSE OF FIG. 7

Thioflavine S Magnification ×1200

Appearance under fluorescent light; the Negri bodies, stained with thioflavine S, are strongly fluorescent and stand out clearly against the dark background of the preparation.

low magnification and examine with high magnification, using glycerol or liquid paraffin as immersion liquid.

The Negri bodies are at once conspicuous by their vivid fluorescence (see Fig. 7 and 8) and brilliant azure-blue colour. The background of the preparation is pale-yellow, the cellular cytoplasm brilliant yellow, and the nucleoli and erythrocytes pale blue.

It should be noted that this fluorescence method, which takes advantage of the affinity of the Negri bodies for fluorescent stains, is quite different in principle from immuno-fluorescence methods (see chapter 5, p. 59), where the staining is brought about by specific fixation on the infected cells of antibodies coupled with the fluorescent substance.

Other Staining Methods

Among the innumerable methods that have been recommended for staining Negri bodies the following also give good results :

(1) Lentz's method (1907) : this is a variation of Mann's method.

(2) Gallego's method (1925), using acid ferric chloride as mordant, followed by staining by Ziehl's method and with picro-carmine.

(3) Romanowsky-Giemsa method, using a buffered Giemsa stain and following the technique of Lillie & Pasternack (1936).

(4) Schleifstein's method (1937) : a combination of Sellers' method with rapid dioxan embedding (see also page 53).

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THE FLUORESCENT ANTIBODY TEST

DONALD J. DEAN [1]

The fluorescent antibody test is the most accurate microscopic test presently available for the diagnosis of rabies and should be employed by all laboratories undertaking such work. Considerable experience, more or less constant practice, and satisfactory reagents and equipment are required. In competent hands the test is fast, comparatively inexpensive, and more accurate than either the examination of films or sections by recommended procedures or mouse inoculation tests. Fresh, frozen, or glycerolated material may be examined. Diagnosis can be made accurately in most instances in minutes or hours, whereas it takes days or weeks with sections or animal inoculation. In our laboratory, for example, specimens involving human exposure received by early afternoon are examined and results customarily reported the same day; results of tests on specimens received during the late afternoon are reported either the same day or the following morning.

Fundamental Principles

Although fluorescent microscopy is not new, its use in immunofluorescence to detect reactions between antigen and its antibody stems from the work of Coons & Kaplan (1950); their techniques were subsequently adopted for use in rabies by Goldwasser & Kissling (1958). Immunofluorescence consists of labelling antibody with a fluorescent dye, allowing the labelled antibody to react with specific antigen if present, and observing the product of the reaction under the fluorescence microscope. A substance is said to fluoresce if, upon absorbing light energy at a certain wavelength, it emits light of another wavelength.

Antigens reacting with antibodies tagged with fluorescein isothiocyanate, the dye most frequently used with rabies, appear under ultraviolet light as brightly coloured, apple-green or greenish-yellow objects against a dark or bluish background, which may or may not contain nonspecific fluorescing material. The character and intensity of the colour may be modified by the use of filters. Fluorescein isothiocyanate is available commercially in powdered form and may be stored for long periods without deterioration.

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The fluorescence microscope consists of a standard microscope of satisfactory quality equipped with a dark-field condenser and an ultraviolet light source.[1] Since only a small portion of the incident radiant energy is converted to fluorescent light, examinations should be made in a darkened room employing the most intense source of light available ; both excitation and barrier filters are used. Although monocular microscopes are preferable, binocular microscopes of satisfactory quality may be used in production laboratories where large numbers of specimens are examined daily. Light used for excitation is of shorter wavelength than that emitted by the preparation. Blues and greens are excited only by ultraviolet light, while yellow and red fluorescence may also be excited by blue-violet light.

Several manufacturers make complete outfits acceptable for fluorescence microscopy and conversion units are available for converting standard microscopes of satisfactory quality for fluorescence work. Fluorescence microscopes made by different manufacturers should be carefully evaluated or the advice of unbiased persons sought prior to purchase. In this laboratory, we are at present using a Zeiss microscope, model GLF658-632, equipped with a model HBO 200, type L2 Osram high-pressure mercury burner ; 10× oculars ; 12.5×, 25×, 40×, and 100× (oil immersion) panchromatic objectives ; and a dry substage dark-field condenser. A drop of oil between the slide and the front lens of the condenser is required when other than a dry substage dark-field condenser is used. The microscope is equipped with multiple excitation and barrier filters. We use filter No. UG5 (467881) in the former and filters 0 and 44, respectively, in the upper and lower filter disks of the latter.

Methods and Procedures

Conjugate

Excellence in fluorescence microscopy is dependent upon the quality of the conjugate used. Although satisfactory commercial conjugates are available, we use sera from hamsters immunized according to schedules developed by the California State Department of Health. All lots of sera to date have produced conjugates superior to those of commercial origin tested and to those prepared from other species in this laboratory. Briefly, conjugate is prepared by fractionating sera by half-saturation with ammonium sulfate, labelling the resulting globulin solution with fluorescein isothiocyanate and passing tagged antibody through a medium or coarse Sephadex G-50 column ; absorption with mouse liver or mouse brain powders is not usually required when a Sephadex column is used. Maximum stability is assured by freeze drying in ampoules containing 0.5-1.0 ml and storage at —20°C. Such products have been kept for at least two years

[1] For a general review of fluorescence microscopy see Price & Schwartz (1956).

without appreciable loss of potency, whereas gradual decay occurs in conjugates stored without lyophilization. Nonspecific fluorescence, a particular problem with many conjugates used at low dilutions, is minimized or eliminated since satisfactory staining is regularly obtained with conjugate diluted 1 : 35 to 1 : 50. Since commercial conjugates of improving quality are available the preparation of conjugate should be attempted only by laboratories experienced in this type of work.

As needed, lyophilized conjugate is removed from the deep freeze and reconstituted to its original volume with sterile distilled water. Centrifugation prior to use is not usually required. The desired amount of reconstituted conjugate, or supernatant if centrifugation is required, is added to the appropriate volume of normal or infective mouse-brain suspension prepared with egg yolk as later described. For example, if satisfactory staining is obtained with a final dilution of conjugate of 1 : 40, 0.1 ml of conjugate is added to 3.9 ml of mouse-brain suspension. The remaining undiluted conjugate is stored at 4°C until used. After such storage, the conjugate may have to be centrifuged lightly to remove precipitate before being added to the mouse-brain suspension. Diluted conjugate may be used for periods of a week or so, provided that precautions are taken to safeguard against undetected decay of product or contamination. Non-rabies antibodies should not be present in conjugate.

Control slides

Control films or smears are usually prepared from the entire brains of mice or young hamsters or from the hippocampus of susceptible animals inoculated intracerebrally with rabies street virus and sacrificed when moribund. Although control films may be stored in acetone, or in a dry condition at —20°C for long periods before use, slides not older than 10 days are to be preferred. *Thin* films are preferable to smears. Uniformity can be achieved by grinding hippocampal or other brain material, without alundum and with or without diluent, to a uniform paste with a mortar and pestle before making the films.

Mouse-brain suspensions

A 20% normal mouse-brain suspension (NMB) for diluting conjugate is prepared using as diluent a 10% suspension of egg yolk, obtained from 6- to 7-day embryonated eggs, in saline phosphate buffer (SPB), pH 7.6. After centrifugation for 10 minutes at 1000 *g* (optional with normal mouse brain but required with infected mouse brain), the supernate is dispensed in glass containers in aliquots sufficient for one day's to one week's work and stored at a temperature of —20°C or lower until used. Suspensions of infected mouse brain (IMB) are similarly prepared using brains from young adult mice inoculated intracerebrally with a 1 : 100 to 1 : 1000 suspension of the CVS strain of fixed-rabies virus and harvested when moribund.

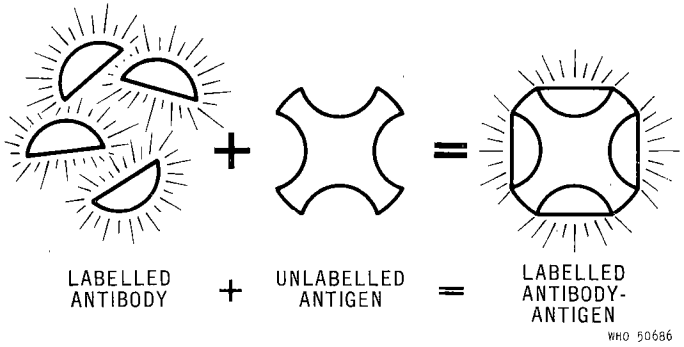
Immunofluorescent staining

Many variations have been developed in the procedures for immunofluorescent staining, including the direct method, the indirect method, and complement staining.

The direct test

The direct method, depicted schematically in Fig. 1, consists in applying a suspension of rabies antibody, previously labelled with fluorescein isothiocyanate, directly to films, impression smears, or sections of tissue

FIG. 1. DIRECT METHOD OF DETECTING RABIES ANTIGEN



Schematic representation of reaction of unlabelled rabies virus (antigen) with fluorescein-tagged antibody to yield fluorescein-labelled antigen-antibody product.

under examination. It works well with brain, salivary gland, tissue culture and similar materials. Best results are obtained when *thin*, uniformly-spread films are prepared on pre-identified glass slides according to the methods for the preparation of films or smears for use with Sellers' stain (see page 34). Glycerolated specimens should be washed twice in saline before use. Not less than four films of salivary gland and/or central nervous system material per specimen should be examined before reporting field specimens as being fluorescent negative; they should include two made from the hippocampus and two from a paste prepared by grinding without diluent or alundum in a mortar and pestle equal portions of material from the hippocampus, the cerebellum, and brain stem from the region of the pons. Component parts of the brain may be examined separately if desired. When the mouse test is used, the remaining ground tissue is diluted with distilled water containing 5% of serum to obtain a 10% tissue suspension and then injected intracerebrally. A direct comparison of the results of fluorescence and mouse inoculation is thus obtained.

Films are air-dried, placed in a Coplin jar or other suitable container, covered with cold acetone [1] and held in a deep freeze at —15° to —20°C for 2-4 hours; excellent results are also obtained with overnight fixation. After fixation, slides are removed from the acetone and placed in the freezer to drain and dry. When dry, slides to be stained are removed from the freezer, air dried to remove condensation and, if films are used, two suitable uniformly thin areas one inch (2.5 cm) long delineated on the slide with a wax marking pencil. Impression smears, two per slide, are similarly demarcated. Slides, including previously prepared controls, are next placed on glass rods strategically arranged across the top of a small sterilizing tray.

One area of film or one impression smear is stained by placing two drops of the conjugate diluted as required in NMB within the area marked by the wax pencil. The other film or smear is stained in the same way with conjugate similarly diluted with IMB. Conjugate should be spread uniformly, without disturbing the film, either by rotating the slides or by means of an applicator stick or toothpick. A fresh stick should be used for each area stained. The container holding the slides is then filled with water at 37°C, covered, and placed in an incubator at 37°C for half an hour.

After incubation, slides are removed from the incubator and their surfaces flooded and washed with SPB, pH 7.4; they are then placed in a rack, immersed in SPB solution for 10 minutes, and air dried in a vertical position. When dried, one drop of 50% buffered glycerol (pH 7.6) is added and cover glasses mounted over the areas to be examined. When properly stained, the positive control film and unknown films containing rabies antigen will contain brilliantly fluorescing apple-green or greenish-yellow structures, varying in size from tiny bodies, which are commonly called sand or dust and are barely visible, to those comparable in size to Negri bodies (Plate II, A & B). Fluorescing nerve fibres and masses of antigen of diagnostic significance are commonly observed in preparations from both the central nervous system and the salivary glands (Plate II, C to F). Control slides, prepared from known infected brain material and stained with conjugate diluted in normal mouse brain, should *always* be examined before and after scanning test films to ensure that the equipment is operating satisfactorily and that films are properly stained. When large numbers of slides are examined consecutively, control slides should also be interspersed with the unknown slides, e.g., every tenth slide.

The staining of films or smears with conjugate diluted with IMB as described above is useful for determining specificity of fluorescence, thus minimizing the reporting of false positives.

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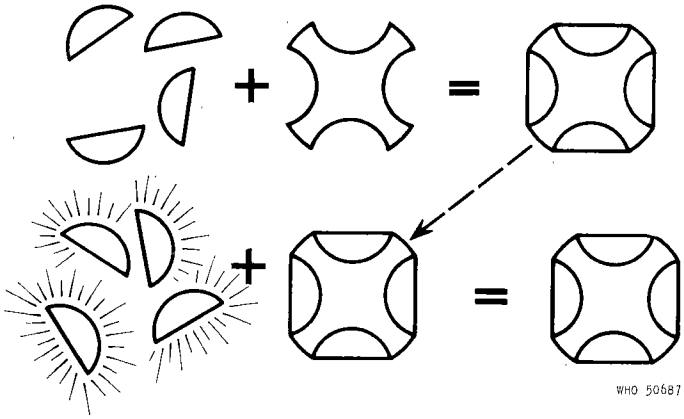
[1] Goldwasser (personal communication) has reported excellent results in preliminary studies with films fixed for 20-30 minutes in methanol held in a container immersed in a dry-ice bath. Field specimens have not yet been processed by this method.

Inhibition of fluorescence staining

The inhibition test (Fig. 2) is used in rabies largely to check specificity of staining, but may also demonstrate antibody in serum. In this test, homologous unlabelled antibody is added to films or tissue sections containing rabies antigen, resulting in an antigen-antibody reaction with unlabelled antibody. When labelled antibody is added, immunofluorescence staining is eliminated or inhibited as compared to films containing antigen not pretreated with unlabelled antibody. Interpretation of results of inhibition tests requires considerable experience. While occasionally useful, the test is not often required in diagnostic work by experienced operators using satisfactory conjugate. Variations of the inhibition test are possible, including the simultaneous labelling of antigen with a mixture of labelled and unlabelled antibody.

FIG. 2. INHIBITION OF FLUORESCEIN STAINING

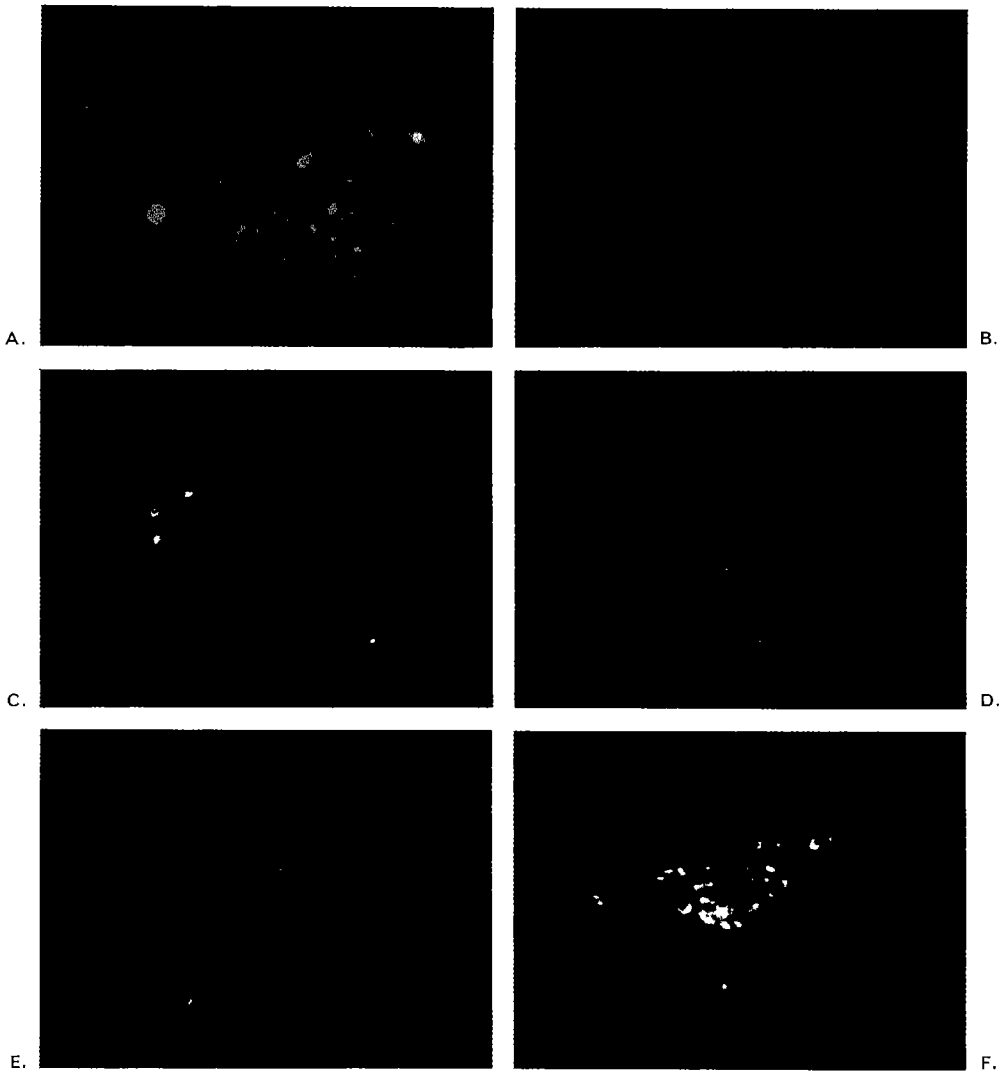


Stage 1 depicts the reaction of unlabelled rabies virus (antigen) with an excess of unlabelled antibody to yield unlabelled antigen-antibody product. Stage 2 represents the reaction between this unlabelled antigen-antibody product and labelled antibody resulting in a second unlabelled product and inhibition of staining.

The indirect test

The indirect test, first described by Weller & Coons (1954) and adapted to rabies by Goldwasser & Kissling (1958), has been used both to detect antibody in serum and to make quantitative determinations of antibody. It may also be used to identify rabies antigen, although the need to use species specific conjugate limits its usefulness in routine diagnostic work. As illustrated schematically with positive serum in Fig. 3, serum under test is added to unlabelled rabies antigen; if homologous antibody is present, an unlabelled antigen-antibody product results. The unlabelled antigen-antibody product is treated with fluorescein-labelled antiglobulin conjugate

PLATE II



Photomicrographs (×500) of brain material treated with hamster conjugate diluted 1 : 5 instead of 1 : 40 (as in the usual method), thus intensifying the bluish background. Note lack of non-specific fluorescence.

A. and B. Typical fields showing fluorescein-tagged antigen varying in size from small, barely discernible particles, referred to as dust or sand, to particles the size of Negri bodies. All have diagnostic significance.

C. Note comet-like appendages of larger particles of fluorescing antigen and thread-like fluorescing object believed to be a nerve fibre.

D. Note extreme variations in size and shape of specifically fluorescing objects.

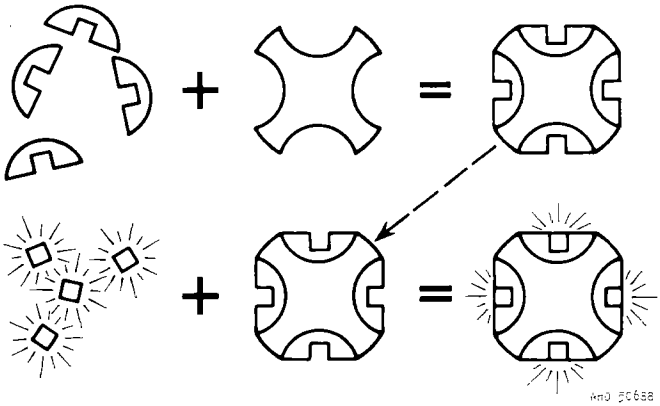
E. Note particularly accumulation of specific fluorescein-tagged antigenic particles within structure believed to be a nerve fibre.

F. Massive accumulation of specific fluorescein-tagged antigen of varying sizes and shapes.

specific for the species from which the test serum was obtained, e.g., anti-human conjugate if the test serum is of human origin, etc. Specific fluorescence indicates the presence of antibody in the test serum. Antibody may be determined quantitatively by testing appropriate dilutions of serum.

The indirect test is more complex and less well developed than the direct test; hence, several modifications have been reported and more can be expected. Even in its present state, however, the test can be completed within one day and offers promise as a screening test and for use in quantitating antibody. As used by Thomas et al. (1963), serum is inactivated by heating at 56°C for 30 minutes and aliquots are then diluted 1 : 5 or as desired in NMB and IMB suspensions; the resulting preparations are incubated at 37°C for 30 minutes prior to use. Standardized slides containing antigen, similar to those used as controls for the direct test, are employed

FIG. 3. INDIRECT METHOD OF DETECTING RABIES ANTIGEN



Stage 1 depicts the reaction of unlabelled antibody from a known species with unlabelled rabies virus (antigen) to yield an unlabelled antigen-antibody product. Stage 2 represents the reaction between labelled antibody from the same known species and the unlabelled antigen-antibody product from stage 1 to yield a fluorescein-stained end-product.

with two areas of the film or the two impression smears delineated with a wax marking pencil. One area of the film or one smear is overlaid with a drop of the serum NMB suspension and the other similarly treated with the serum CVS suspension. Controls include similar films treated with known positive and negative sera previously determined by the results of serum neutralization tests or the indirect fluorescent antibody test. Films or smears prepared from normal brain material are included in each test to measure specificity of staining.

Following incubation at 37°C for 30 minutes, slides are washed in SPB (pH 7.2) for 10 minutes, air dried, and the films or smears overlaid with a suspension consisting of one part of anti-species conjugate, three

parts of appropriately diluted rhodamine counter-stain, and four parts of NMB suspension. Films are again incubated at 37°C for 30 minutes, washed and dried as above, mounted with cover slips using buffered glycerol (90% neutral glycerol, 10% SPB, pH 8.5) and examined by fluorescence microscopy. Sera are considered positive if controls are satisfactory and if specific fluorescence can be demonstrated in films treated with the unknown serum suspended in NMB but not in those treated with the serum CVS preparation.

Immunofluorescence should be observed in control films containing antigen stained with positive serum suspended in NMB but not in those suspended in IMB or in those treated with negative serum incorporated in NMB or IMB. Films prepared from non-infective mouse brain treated with positive serum suspended in NMB and IMB should not give specific fluorescence.

A third procedure, complement staining, is somewhat similar to the indirect procedure and utilizes the ability of the antigen-antibody product to fix complement. This procedure has not been widely used in rabies.

Discussion

Refinements in fluorescence will continue to be made, particularly in regard to light source, conjugate quality, and staining techniques. Current tests, however, have wide application and immunofluorescence staining has eliminated the problem previously encountered with conventional stains of differentiating non-rabies viral inclusions or artefacts from Negri bodies.

Fluorescent-positive, mouse-negative specimens can occasionally be expected since the fluorescent antibody test detects killed or inactivated antigen as well as live virus. In this respect, fluorescence is more sensitive than tests in mice. More worrisome is the fact that many laboratories continue occasionally to encounter specimens that are negative on fluorescence but positive on mouse inoculation. A recent survey of state public health laboratories in the USA using fluorescence for the diagnosis of rabies revealed a total of 99 specimens in 1963 which were negative for fluorescent antibody but positive in mice, which suggests that the test is not foolproof. Fortunately, such results apparently occur most frequently in laboratories that have not yet acquired adequate experience with fluorescence. It is perhaps significant that two major laboratories—New York and California —reporting almost perfect correlation in recent years, use conjugate of hamster origin that can be diluted as much as 1 : 50 prior to use, thus minimizing or eliminating the problem of non-specific fluorescence ; both laboratories experienced difficulty before this conjugate was used. For three years, our laboratory has found perfect correlation between fluorescence and mouse tests. The factors believed most responsible for this

performance are greater competency resulting from continued experience, use of improved conjugate, and better sampling. However, we continue to test in mice all fluorescent-negative specimens involving human exposure and recommend that others do the same until satisfied that the mouse test is no longer needed.

Errors may also be due to the use of unsatisfactory or inadequately adjusted equipment, nonspecific fluorescence, gradual and unrecognized loss of light from a deteriorating bulb, failure to use adequate controls, and colour blindness on the part of the operator. On one occasion, washing multiple slides in a common container resulted in tagged antigen being washed off a rabies-positive specimen and becoming attached to another slide, making an otherwise negative specimen positive.

Adequate sampling is an important problem. Since rabies virus is usually transmitted from the site of inoculation to the central nervous system via the peripheral nerves (Dean et al., 1963), we routinely examine brain-stem material either separately or as part of the composite sample. On repeated occasions, the brain stem has been positive when films prepared from the hippocampus, cerebellum, or cerebral cortex were negative. Brain-stem sampling is particularly advantageous when animals are killed early in the course of the disease.

With satisfactory conjugate, counterstains such as rhodamine-conjugated albumin are rarely needed. When rhodamine is used in dilutions of less than 1 : 80, loss of specific fluorescence occurs in direct proportion to the concentration of rhodamine used. Dilutions of 1 : 80 or greater are apparently sufficient to stain background material a light brick red leaving the greenish-yellow rabies antigen in sharp contrast. There is no apparent correlation between the condition of the specimen examined and the degree of autofluorescence. Good to excellent fluorescence with little or no autofluorescence has been observed in badly decomposed specimens and, occasionally, autofluorescence is encountered in fresh specimens. With experience, however, one is able to differentiate between specific and nonspecific fluorescence without great difficulty.

Examination of the salivary glands should be made routinely in the case of bats involved in human exposure and may yield valuable information with other species as well. Salivary glands are more difficult to sample adequately and to process than brain material. Since grinding with mortar and pestle is not usually satisfactory owing to the character of the tissue involved, films are customarily prepared from the cut surface or surfaces of the gland by the impression smear technique and stained as with brain material used for the direct microscopic test. Multiple films should be examined before a specimen is reported as negative. Autofluorescence can be a particular problem. However, we have encountered little difficulty with salivary gland material, provided that sampling is adequate and care is taken to make satisfactory films. Perfect correlation has been obtained between

the results of mouse inoculation and fluorescent antibody tests in the more than 100 specimens examined to date.

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MOUSE INOCULATION TEST

HILARY KOPROWSKI [1]

The mouse inoculation test, in spite of its simplicity, depends greatly on the accuracy of its performance for dependable results.

Choice of Mice

Strain

Usually white mice of any breeding strain may be considered suitable. Preference should be given to the Swiss albino strain since it is very susceptible to rabies virus and it is easy to maintain the breeding stock in the laboratory. If the Swiss albino stock is not available, however, almost any breed of mice, except grey wild mice, can be used, because a genetically resistant strain has not yet been found. Grey wild mice should be excluded, not because of insusceptibility but because of the difficulty in restraining these animals in cages during the observation period.

Age

Mice of all ages are susceptible to intracerebrally introduced rabies virus. However, it is easiest to inoculate, maintain, and observe mice which are 21-35 days old (8-12 g in weight) at the time of inoculation. There is some indication that the suckling mouse is somewhat more sensitive to rabies virus than the adult mouse.

Sex

Mice of both sexes are equally susceptible to rabies virus. It is inadvisable to keep older mice of the same sex in one cage since they are apt to kill each other in fights before the observation period is completed. This applies especially to males.

General health

It is imperative that the animals chosen for inoculation be in good health. It is important to know the history of the breeding colony, and it is advisable to inspect the animals closely before inoculation.

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[1] Director, Wistar Institute, Philadelphia, Penn., USA.

Ectoparasites on the animal, ruffled fur, and unmistakable signs of diarrhoea, should disqualify the animals immediately. If the mice are sent to the laboratory from a considerable distance, it is advisable to postpone inoculation for a minimum period of three days in order to let them rest and become adjusted to changed conditions. In such cases it may be equally important to leave a few animals uninoculated in order to observe the death-rate among "normal" mice as compared with inoculated animals.

Preparation of Supposedly Infectious Material for Inoculation

Choice of tissue

Either the brain or the salivary-gland tissue of a suspectedly rabid animal may be used for virus isolation. Detection of the virus is more frequently possible in the brain than in the salivary gland. However, from the epidemiological and epizootiological points of view, it is important to examine the salivary glands for the presence of virus.

Although it is relatively immaterial which part of the brain tissue is chosen for the preparation of the suspension, preference may be given to Ammon's horn, the cerebellum, and parts of the cortex. When salivary-gland tissue is chosen, the submaxillary glands should be considered as those most likely to show the presence of rabies virus. Also, when salivary-gland tissue is used, it is always advisable to mince it before grinding.

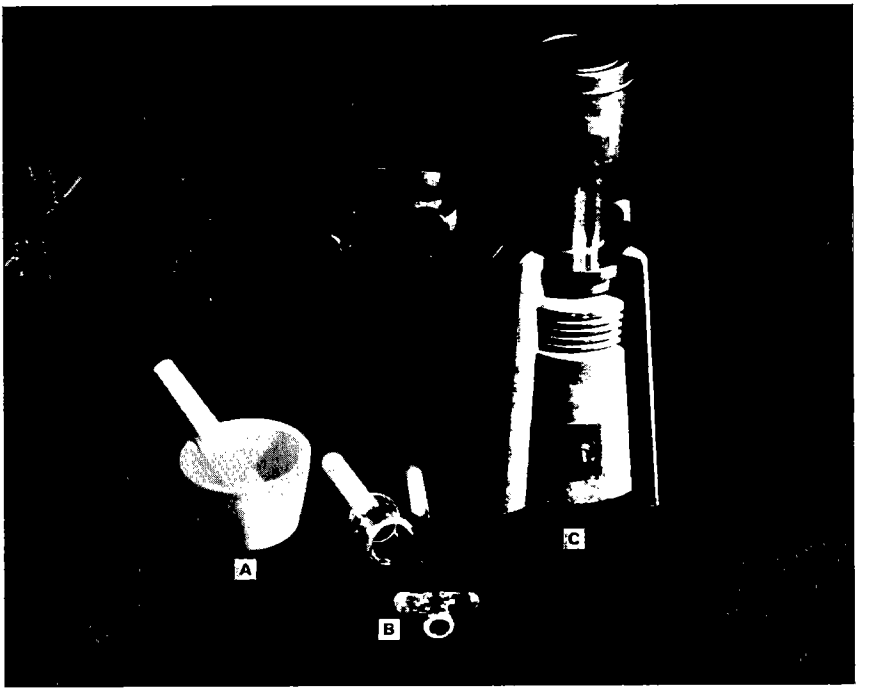
Grinder

The choice of grinder depends to a certain degree on the amount of tissue available. If more than 3-4 g of material are available, a small Waring blendor should be preferred over any other type of grinder (see Fig. 1). However, if the amount of tissue is less than 3 g (which is more usually the case), or if a Waring blendor is not available, the following grinders or grinding devices may be suitable in the order of preference listed.

(*a*) *TenBroeck grinder* (see Fig. 1) : This grinder is a handy tool, easy to assemble, manipulate, clean, and sterilize, but only brain tissue may be used in it since salivary-gland tissue is too tough for it to grind properly. A slight disadvantage is its fragility. If improperly used, it may break while being manipulated ; and the grinder may be easily damaged during cleaning, sterilizing, etc. From the point of view of personal safety, the Waring blendor and the TenBroeck grinder, if properly used, have some advantage over the other grinding devices.

(*b*) *Mortar and pestle* (see Fig. 2) : This is a time-honoured method of grinding which has one advantage, namely, that with the help of an abrasive (e.g., sterile sand) even the toughest tissue can be properly ground up.

FIG. 1. GRINDERS FOR PREPARATION OF TISSUE



A = Mortar and pestle B = TenBroeck grinder C = Waring blendor (small size)

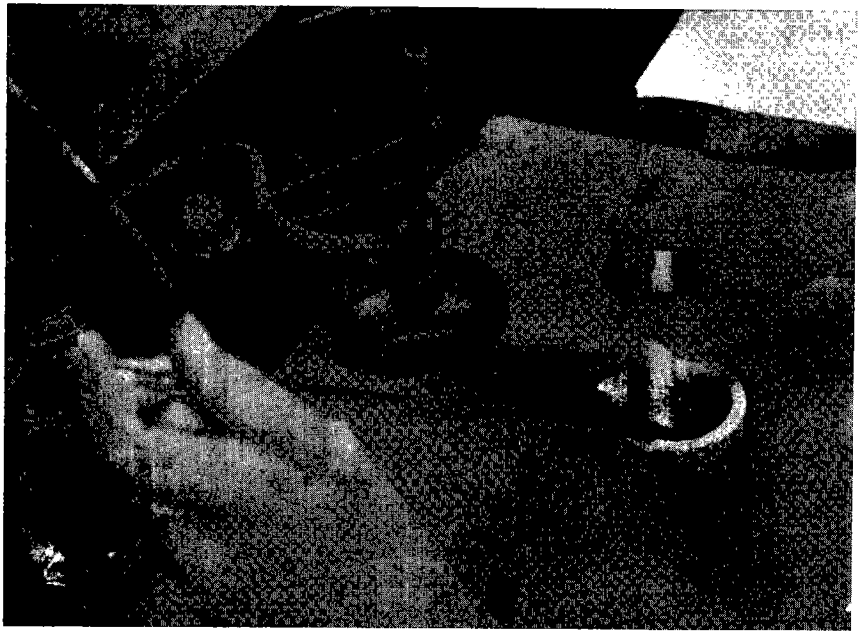
The mortar and pestle cannot be operated under such sterile conditions as can the Waring blendor or the TenBroeck grinder; it can, however, be easily cleaned and sterilized, and stands wear and tear for a long period of time.

Diluent

The choice of diluent may be left to the user but it is preferable to use an isotonic salt solution. However, mice have been known to withstand easily an intracerebral inoculation of sterile distilled water. The following diluents may be considered in order of their availability:

(*a*) *A physiological salt solution containing varying amounts of animal serum (10-50% concentration)*: This is by far the commonest diluent used. It should be carefully ascertained, however, that the donor animal has never been vaccinated against rabies. It is therefore advisable to avoid the use of dog, cat, or cattle serum. Normal sheep serum seems to possess some "antiviral" properties which are absent from rabbit serum. Thus, if it is available, the rabbit should be considered as the first source

FIG. 2. PREPARATION OF TISSUE SUSPENSION BY EMULSIFICATION WITH MORTAR AND PESTLE



By courtesy of United States Department of Health, Education and Welfare, Public Health Service, Communicable Disease Center (US DHEW-PHS-CDC)

of blood. It is preferable to inactivate the serum for 30 minutes at 56°C before using it as a component of the diluent. Diluent containing serum can be sterilized only by filtration through bacteria-retaining filters.

(*b*) *Other diluents* :

(i) skimmed milk ;
(ii) bovine serum albumin in buffered salt solution ;
(iii) physiological salt solution in distilled water.

These are not particularly recommended, although rabies virus seems to be relatively non-susceptible to the inactivating action of salt solution, in contrast to such viruses as eastern or western equine encephalomyelitis.

Note : If a tissue suspension is to be frozen and stored, a 50% solution of serum in water should be the preferred diluent.

Bacterial sterility

There is no need to add antibiotics to suspensions of *brain tissue* if the material has been handled with reasonable precautions at the autopsy

and dispatched in a sterile container. A 50% glycerol solution is highly recommended for preserving the material since, in addition to its preserving qualities, it exerts a strong bacteriostatic action (see chapter 3, page 40). If there is any doubt, however, it may be safer to add enough streptomycin and penicillin to the suspension to obtain a concentration of 2 mg of streptomycin and 500 IU of penicillin per millilitre of the final suspension. If antibiotics are added, it is best to let the suspension stand for at least 30 minutes before inoculating the animals.

It is always advisable to add antibiotics to suspensions of *salivary glands*. Regardless of the addition of antibiotics, the salivary-gland tissue suspension should always be cultured for possible bacterial contamination. Beef-infusion broth, thioglycolate medium, and blood-agar are considered to be good culture media for this purpose. If bacterial growth is observed, an attempt should be made to identify the bacterial agent. If the results of the mouse inoculation test are equivocal (see page 79) it may be advisable to test the pathogenicity of the bacterial contaminant for mice by intracerebral inoculation.

Concentration of infected tissue in the suspension

This is optional. If the suspension is for storage, it is advisable to prepare a 20% suspension by weight. The weight of the tissue in grams multiplied by 4 gives the required amount of diluent in millilitres. However, if the suspension is to be used for inoculating mice intracerebrally, a 10% suspension by weight should be given preference. Either dilute the 20% suspension by adding an equal volume of diluent, or prepare the suspension by multiplying the weight of tissue in grams by 9 in order to obtain the required amount of diluent in millilitres.

In attempting to isolate certain strains of rabies street virus, the "self-sterilizing neuro-infection" phenomenon may be suspected on very rare occasions, and in such cases it is advisable to dilute the tissue suspension more than 10% (see pages 79 and 80).

Centrifugation and filtration

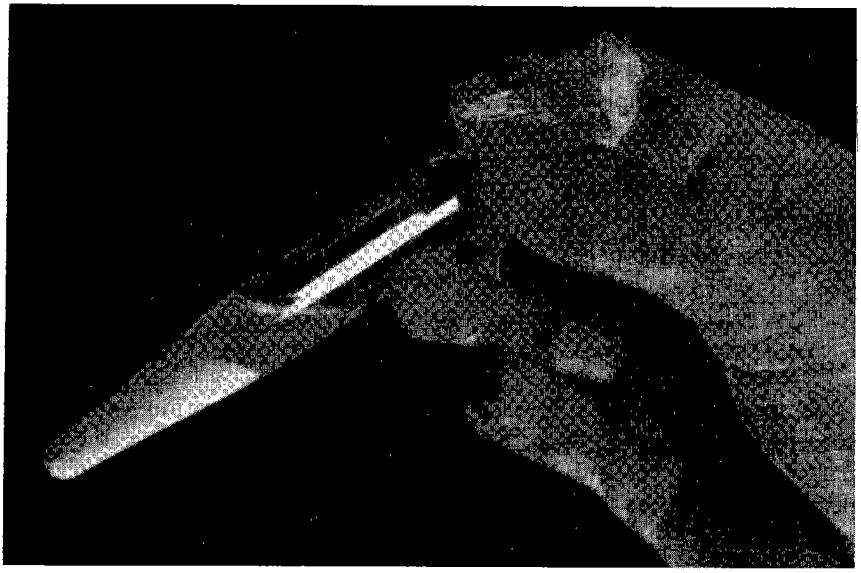
If the equipment is available, it is advisable to centrifuge the tissue suspension for 5 minutes at 1000 rev/min in order to remove the gross particles (see Fig. 3 and 4). However, if no centrifuge is available, it is perfectly feasible to inject mice intracerebrally with a 10% uncentrifuged brain suspension. Salivary-gland suspensions, if uncentrifuged, must be filtered through one or two layers of sterile gauze in order to prevent the death of the animals from trauma.

FIG. 3. CENTRIFUGATION OF TISSUE SUSPENSION

By courtesy of US DHEW-PHS-CDC

1000 rev/min for 5 minutes

FIG. 4. APPEARANCE OF CENTRIFUGED TISSUE SUSPENSION



By courtesy of US DHEW-PHS-CDC

Inoculation of Mice

Choice of syringe

Syringes should be chosen which can measure accurately 0.03 ml (single mouse dose). Thus $^1/_4$-ml tuberculin syringes should be considered first, followed by ½-ml or 1-ml tuberculin syringes. For intracerebral inoculation, 27- or 26-gauge (0.40-0.45-mm) needles 1-1.5 cm long should be selected. Larger-gauge needles cause trauma to the brain substance.

Anaesthesia

It is strongly recommended that mice be anaesthetized before they are inoculated : inhalation of ether is the best form. A battery jar with a specially fitted wire bottom may be used for the purpose (Fig. 5). If no such device is available, pentobarbital sodium injection should be considered. It is a good procedure to have the work-table used for mouse inoculation drawn well away from the wall, so that an assistant can etherize the mice from a position opposite the operator.

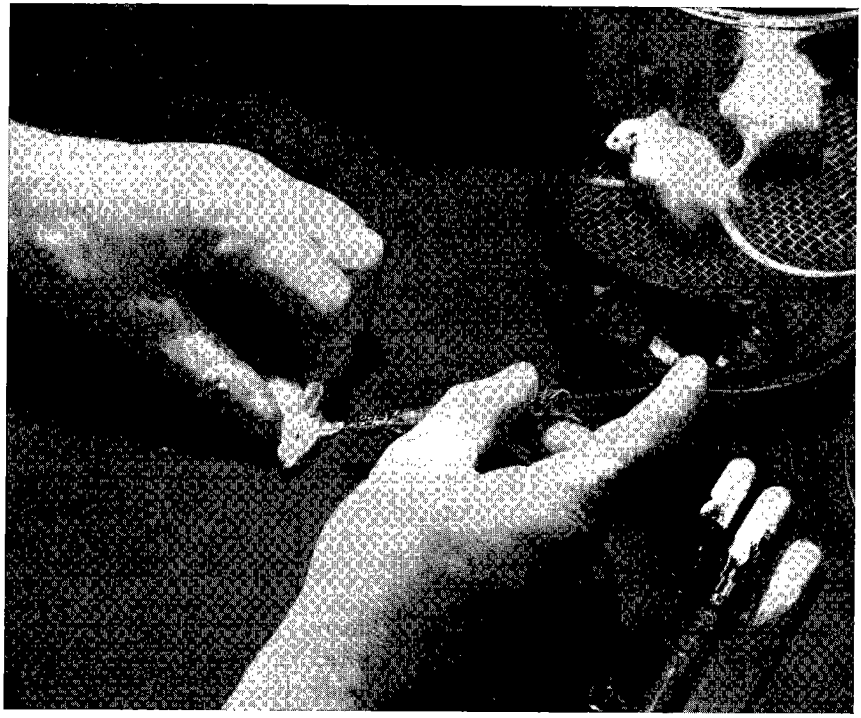
Inoculation technique (see Fig. 5)

There is a wide choice of methods of inoculation. The present author, who is right-handed, prefers the following technique.

Anaesthetized mice are laid on the left side, the legs pointing towards the inoculator. With the thumb of his left hand, the inoculator supports the lower jaw of the animal, with his index finger behind the skull of the animal. Pressure should be very slight in order to avoid the asphyxiation and death of the animal. In his right hand, the inoculator holds the syringe in a horizontal position parallel to the table surface and perpendicular to the head of the mouse, with the needle pointing towards the inoculator. With a quick thrust, the needle is pushed through the skull of the animal at the place that can best be described as the apex of an imaginary angle the arms of which point to the animal's right eye and right ear. The needle easily penetrates the bone and should then be further inserted for about 0.1-0.2 cm into the brain tissue. When the inoculator uses a 1.5-cm needle, he should be careful not to penetrate too far since he may then end by giving the injection into the base of the skull. The plunger is pushed to the next 0.03-ml mark and the needle is then gently withdrawn. For intracerebral inoculation, move the inoculated mice away from the syringe hand (i.e., right-handed persons pass the inoculated mice to the left). This is to prevent crossing hands, which may result in catching a finger on the needle held in the opposite hand.

The inoculated mice are immediately placed in a can or box previously prepared and identified by a tag bearing the mouse-group number or

FIG. 5. INTRACEREBRAL INOCULATION OF MICE



any other particular identification mark. If a large series of inoculations is made, it is worth while checking the number of living mice in each series after the entire experiment is finished. If any animals are found dead, an equal number of new animals should be inoculated and added.

Under no conditions should the same syringe be used for inoculation of two different suspensions. If an adequate number of sterile syringes is not available, each syringe should be boiled between inoculations, and care should be taken to let it cool before filling it with inoculum.

It is well always to use the same rigid precautions to establish good habits in virus work. For example, the rapid emptying of a syringe into a pan of water will produce an aerosol which can cause inhalation infection in the operator, or in the animals with which he is working, if the disease can be transmitted by such exposure. Virus may be spread from table to hands, and if these are not washed properly it is possible to contaminate subsequent specimens during grinding of tissue in a mortar—for example, virus may fall from the hands or sleeves into the mortar. Mice laid on the table after etherization may awake and have to be put back in the ether jar, thus contaminating it. Should other studies be carried out using the same ether jar, virus may be deposited on the head of normal mice

and be carried into the brain by the inoculation procedure. The work-table must be considered contaminated until washed with soap and water. Dilute bichloride of mercury is a good disinfectant for table tops. Cresol and phenol solutions are no better than water for the most common virus contaminant of mouse tissue, namely, the mouse encephalomyelitis virus, and several other viruses; phenol has no appreciable virucidal action on rabies virus.

When inoculation of all the mice is finished, the syringe and needle may be rinsed in water, provided this is done gently with the point of the needle well below the surface of the water in the sterilization tray. Sterilize all syringes and needles by boiling for five minutes.

Observation of Inoculated Mice

Although rabies virus will only rarely cause signs of illness in mice before the fifth day after intracerebral inoculation, it is advisable to check mice daily, beginning with the first post-inoculation day. The number of mice found normal, sick, or dead is recorded on a mouse-history card (see Fig. 6) which remains on file as a permanent record of the experiment. The observation period should extend for a minimum of 21 days after inoculation. Only rarely will rabies virus be detected in the inoculum later than 21 days after the inoculation.

FIG. 6. MOUSE-HISTORY CARD

No. Date Virus

Strain Passage Preparation

Volume Dilution

DATE																						
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1																						
2																						
3																						
4																						
5																						
6																						
Checked by																						

Age of Mice Route

LPR 542

The following signs may be noted:

(*a*) Ruffled fur; (*b*) tremors when held in the air by the tail with a pair of forceps; (*c*) lack of co-ordination of hind legs—note gait when placed on table and made to move; (*d*) paralysis; (*e*) prostration (near

death). Letter symbols denoting the different signs are recorded each day on the mouse-history card.

Deaths in mice occurring 24-48 hours after intracerebral inoculation are attributable to causes other than rabies virus (trauma, bacterial contamination, other viruses). For diagnostic purposes, one or two mice may be sacrificed each day, beginning on the fifth day, and a search made for Negri bodies as well as for the presence of rabies antigen as demonstrated by immunofluorescent antibody staining. Frequently, an early diagnosis is thus obtained, particularly in instances where certain strains of street virus might take between 1 and 3 weeks to kill the mice.

Note : Clinical signs of illness in inoculated mice cannot be considered characteristic of rabies. Although signs of paralysis 5 days or more after inoculation may give grounds for suspecting the presence of rabies virus, the same signs may be observed in numerous other viral, bacterial, and protozoal infections which involve the central nervous system of the mouse. Definite evidence of the identity of the virus is obtained with the serum-virus neutralization test (see chapter 7, page 81).

Further Passages of Infected Material

If it is desired, brain tissue from mice that have succumbed to infection after inoculation with the original virus may be made into a suspension as described above ; it can then be stored, or used in neutralization tests, or inoculated into another group of mice.

Removal of Mouse Brain

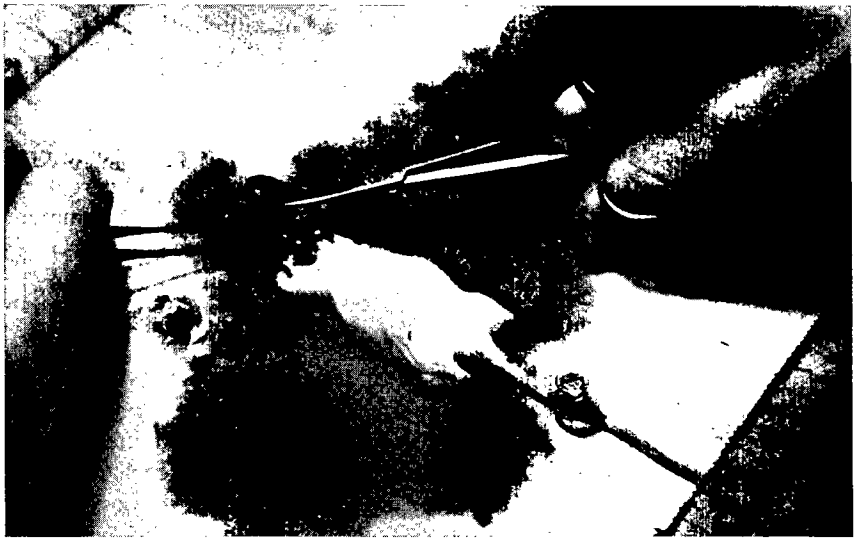
The brains of all mice that have died during the night, as well as the brains of those mice that have been sacrificed when prostrate, should be removed for Negri-body detection and demonstration by immunofluorescent antibody staining (see chapters 3 & 5, pages 26 and 59). The mice showing symptoms of prostration (terminal symptoms of rabies) should be killed with chloroform.

The dead mouse should be pinned to a dissecting board with the dorsal aspect of the body facing upward. Only two pins are necessary, one through the nose and one through the base of the tail (see Fig. 7). Three pins or spring clips may also be used : one through each of the forelegs, and one through the back of the tail.

After disinfection with ethanol, the skin of the head and neck is cut away with forceps and scissors, exposing the skull. The skull is grasped in the orbits with mouse-tooth forceps and the calvaria of the skull is cut away with curved scissors, thereby exposing the brain. The brain is removed with curved scissors (see Fig. 7) and is transferred to a sterile Petri dish.

A thin section of the brain is cut out, just anterior to the cerebellum, and is transferred to a wooden tongue-depressor or a paper towel. A clean microscope slide is then pressed lightly against the cut surface of the section; the pressure should be sufficient to create a slight spread of the exposed surface against the glass slide. Negri-body stain and immunofluorescent antibody stain should then be applied to the slide.

FIG. 7. REMOVAL OF MOUSE BRAIN



By courtesy of US DHEW-PHS-CDC

Complications of the Mouse Inoculation Test

Bacterial contamination of inoculum

If bacterial contaminants have caused the death of the mice in spite of the addition of antibiotics, and if the original suspension has been preserved, the following methods may be tried in an effort to overcome the interfering action of the bacteria.

(*a*) *Filtration through bacteria-retaining filters :* The supernatant liquid of a suspension centrifuged at 1500 rev/min for 15 minutes should be used for this purpose. Since rabies virus is a fairly large particle and since, in general, the concentration of virus in specimens submitted from the field is not very great, the virus may be lost in the process of filtration.

(*b*) *Dilution method :* Sometimes the suspension can be diluted beyond the end-point of bacterial contamination with retention of viral activity, but this happens very rarely.

(*c*) *Prolonged storage :* In some instances, it is easier to combat bacterial contamination after the tissue suspension has been stored for a period of time either at freezing temperatures or in glycerol (see pages 40 and 73).

(*d*) *Parenteral inoculation :* The Syrian hamster, which is the animal most susceptible to parenteral infection, may be chosen for this purpose. Mice are relatively insusceptible to parenteral infection with rabies.

Presence of two viruses

This is particularly confusing if the second virus has pathogenic properties similar to those of rabies. Again, intracerebral or parenteral inoculation of animal species other than the mouse may be attempted, particularly in view of the extremely wide host-range of rabies virus.

" Self-sterilizing neuro-infections " or interference phenomenon

In certain instances, either because of the properties of a particular strain of rabies virus or because large amounts of inactive virus particles may interfere with the living virus, it may be necessary to dilute the inoculum ten- or a hundredfold, or even more. There is no rule for determining when this should be done. However, if failure to isolate rabies virus is consistently encountered in the same species of animal in a particular geographical area, the possibility of an interference phenomenon should be seriously considered, and tissue suspensions should be diluted beyond 10% concentrations.

CHAPTER 7

THE SERUM-VIRUS NEUTRALIZATION TEST

HARALD N. JOHNSON, B.SC., M.A., M.D.[1]

In order to identify an unknown virus by the serum-virus neutralization test, the virus is titrated in normal serum and in a known virus-immune serum. If the unknown virus is neutralized by rabies-immune serum and not by the normal control serum, it is assumed that the virus under test is a strain of rabies virus. It is important to check all standard stocks of rabies virus by the serum-virus neutralization test to determine whether the stock virus contains viral contaminants derived from the animals or tissue culture systems used as a source of the virus.

Preparation of Stock Virus Suspension

Prepare a 20% suspension of infected mouse brain using a diluent of physiological phosphate saline containing 0.75% of bovine albumin fraction 5, or 2% of inactivated guinea pig, hamster or horse serum. The source of the virus is usually the 2nd mouse passage and if this passage is made by intracerebral inoculation of 1-3-day old suckling mice, the virus titre is usually higher than that observed using adult mice. Place aliquots of 0.5 ml or 1.0 ml in 2-ml ampoules, flame seal, and store at —60°C as stock virus. Prepare serial ten-fold dilutions of this stock virus in a diluent as described above, ending with a dilution of 10^{-7}, and titrate each dilution by intracerebral inoculation into mice in order to determine the dilutions of the virus to be tested in the serum-virus neutralization test.

Serum Specimens

It is essential to use a rabies-immune serum of known potency, previously tested against the virus to show that it possesses specific neutralizing antibody.[2] The standard rabies-immune serum should be stored in the lyophilized state at 4°C or in the frozen state at —15°C or lower in a mechanical

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[2] A known positive serum will be supplied on request by the World Health Organization, Geneva, Switzerland.

freezer. Under such conditions it is not necessary or desirable to use a preservative in the serum. For preparing stock normal and rabies-immune serum, it is best to use hamsters or guinea pigs as a source of serum. It is also desirable to use homologous infected brain tissue as the source of the antigen in immunizing the animals—for example, infected hamster brain tissue for immunization of hamsters. The virus cultivated in hamster kidney tissue culture may be used for the immunization of hamsters, but if the antiserum is to be tagged with fluorescein it is best to use brain tissue as the source of the antigen. Immunization of the animals for the preparation of rabies-immune serum is usually accomplished by giving 4 weekly intraperitoneal injections of betapropiolactone-inactivated rabies CVS strain brain-tissue virus, mixed with an equal volume of adjuvant. Subsequently, 2 injections of active virus are given at 2-week intervals and the animals are then bled at 10 days to 2 weeks after the last dose of virus.

Performance of the Test

Serum

Prepare 2 rows of 13- × 100-mm test tubes, to include tenfold dilutions of 10^{-2} through the expected end-point of the virus. Prepare typed adhesive labels reading N-2, N-3, etc., for the normal serum series, and RI-2, RI-3, etc., for the rabies-immune serum series. In each of the tubes marked N, place 0.2 ml of the normal serum, measured from a 1-ml pipette, graduated in divisions of 0.01 ml. In each of the tubes marked RI, place 0.2 ml of the rabies immune serum. Use a separate pipette for each serum.

Virus

In order to obtain final dilutions of 10^{-2}, 10^{-3} etc. in the serum-virus mixture, prepare ten-fold dilutions (2×10^{-2}, 2×10^{-3} etc.) of the stock 20% virus. When mixed with equal quantities of serum, these will give the correct final dilutions. For preparation of the serial dilutions, it is convenient to use 13- × 100-mm test tubes with screw-cap closures. The screw-cap can be handled without contaminating the palm of the hand. Prepare typed adhesive labels reading St-10-2, St-10-3 etc., the prefix St signifying stock virus. Transfer 2.7 ml of the diluent to each of the tubes labelled St-10-2, St-10-3 etc. To prepare the virus dilutions, transfer 0.3 ml of the stock 20% virus suspension to the tube labelled St-10-2. Discard the pipette. Take a clean 1-ml pipette and mix the suspension by filling and emptying the pipette 10 times ; transfer 0.3 ml to the next tube, labelled St-10-3, and discard the pipette. Subsequently, mix and transfer in this manner until the series is complete, using a clean pipette for each dilution. A routine

should be established whereby the tube to which the virus has been added is moved two spaces along the rack before mixing, so as to make it evident that this mixture is to be pipetted before transfer to the next tube. In taking up a virus suspension in a pipette use gentle aspiration so as to avoid production of an aerosol containing the virus.

Serum-virus mixture

Beginning with the highest ten-fold dilution of the stock virus, transfer 0.2 ml to the normal (N) tube and 0.2 ml to the rabies-immune (RI) tube bearing the same dilution number, in that order. Taking a clean pipette, transfer 0.2 ml of the next ten-fold dilution to the serum tubes bearing the same dilution number, and so on until the series has been completed. Agitate the serum-virus mixture gently and place it in an incubator at 35-37°C for one hour. Thereafter, transfer the rack to a refrigerator at 4°C to chill the serum-virus mixture quickly and hold it at this temperature until required for inoculation into mice. It is not necessary to keep the serum-virus mixtures in an ice-bath during the inoculation procedure, but it is important to avoid exposing them to sunlight or to intense electric light.

Inoculation of Mice

Inoculate each of 4 to 6 young adult mice intracerebrally with each serum-virus mixture, using the technique described in chapter 6 (page 75). Complete the inoculation of the rabies-immune serum-virus before testing the normal serum-virus mixtures. A different syringe and needle should be used for each serum-virus mixture.

Interpretation of Results

The usual observation period for the inoculated mice is 21 days but it is recommended that the mice be held for 30 days because of the long incubation periods observed with some natural strains of rabies virus. The titre of the virus in normal serum and rabies-immune serum is calculated as described in chapter 18 (page 145). The virus-neutralization index is obtained by subtracting the log of the LD_{50} titre of the virus in rabies-immune serum from that in normal serum. If the difference is greater than 2, i.e. if more than 100 times the LD_{50} of the virus is neutralized by the rabies-immune serum, the identity of the unknown virus is established. Mouse-passed strains of brain-fixed rabies virus may sometimes be contaminated with certain natural viruses derived from mice, notably mouse encephalomyelitis virus.

Maximum neutralization of most viruses is usually obtained in tests using fresh serum specimens or serum specimens stored in the frozen or lyophilized state. In large-scale testing of serum specimens from dogs vaccinated against rabies, the author has noted a fairly uniform log difference of 1-1.5 in tests of fresh serum and serum inactivated at 56°C for 30 minutes. Inactivation of serum specimens has come into use to avoid differences observed in tests of the same serum when tested fresh and after storage. Rabies hyperimmune serum as used for identification purposes has usually been stored for some time and the blood serum enzymes have been inactivated, so that it is not necessary or desirable to inactivate the serum.

Strains of rabies virus derived from spotted skunks and bats in North America are very pathogenic for infant mice when inoculated intraperitoneally. The pathogenicity is best preserved by isolation and passage in hamster kidney tissue culture, but 1st or 2nd mouse-passage virus is usually pathogenic for infant mice when inoculated intraperitoneally. Serum-virus neutralization tests using this experimental host system are much more sensitive than the usual mouse-inoculation test for detecting virus-neutralizing antibody in blood-serum specimens.

A different serum virus neutralization test is used for determining the potency of rabies immune serum. In this test, a constant amount of virus is tested against serial dilutions of serum (see chapter 22, page 167).

GEL-DIFFUSION TECHNIQUES

PIERRE LÉPINE [1]

Immunoprecipitation reactions are among the most sensitive tests for the identification of specific antigen and specific antiboby. Studies by Oudin (1946) and by Ouchterlony (1949) on gel-diffusion techniques have led to important applications of immunoprecipitation reactions in the diagnosis of virus diseases. Several papers have been published on the use of these techniques in rabies : with a proper appreciation of the sensitivity of these methods as well as their limitations it is possible, under certain conditions, to make a rapid identification of a rabies virus or a rabies immune serum.

It is necessary, however, that the antigen taking part in the reaction should contain sufficient virus and that the antibody should be present in sufficient quantity if the reaction is to yield a visible precipitate. From this it follows that :

(*a*) *If unknown antibodies are to be identified* (for example, for the diagnosis of latent rabies in dogs in areas where rabies is enzootic), it is necessary to have available a sufficiently concentrated rabies antigen to produce a reaction. In such cases, therefore, the unknown serum is reacted either with an antigen prepared from the brains of suckling mice inoculated with a very virulent strain of high-titre fixed virus or, as described by Grasset & Atanasiu (1961), with a concentrated rabies antigen prepared by tissue culture.

(*b*) *If an unknown virus is to be identified*, it is necessary to have available a hyperimmune serum prepared by immunizing the donor animal with a rabies virus obtained from an animal of a different species from the one under investigation. This case frequently arises when dogs suspected of rabies have been killed and their brains submitted for examination, either to confirm a diagnosis of rabies reached on the basis of a search for Negri bodies or to provide additional evidence when the histological examination has yielded doubtful or negative results. For this type of test, successful use has been made both of the Pasteur Institute serum made by hyperimmunization of horses with rabbit brain (Villemot & Provost, 1958 a, b ; Thiéry, 1960 ; Grasset & Atanasiu, 1961 ; Atanasiu et al., 1963), and of a serum

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made by immunization of donkeys with the Flury strain of avianized virus (Villemot & Provost, 1958 a, b).

Methods and Material

Petri dish method

This method was developed by Villemot & Provost (1958 a, b), who were the first to show the presence of two precipitable fractions in tissues containing rabies virus. The technique consists in punching out small cups in an agar gel poured in a Petri dish and then filling some of these with rabid brain tissue and the remainder with immune serum. Soluble antigen and precipitating antibody diffuse towards one another through the gel and react to give a precipitate, which forms two lines clearly visible to the naked eye.

Medium

Bacto-Agar	15 g
Methyl orange	0.03 g
Merthiolate	0.2 g
Water	1000 ml

The methyl orange facilitates the reading of the results and the merthiolate inhibits bacterial growth.

The medium is dispensed in 30-ml portions in 22-mm tubes and stored at 4°C. When required, the tubes are placed in the water-bath and the melted medium poured into scrupulously clean Petri dishes. After cooling, the medium should be perfectly homogeneous and clear, and should be free from all particulate matter. Of the stains that have been tried, methyl orange seems to have given the best results, while 0.012% Congo red has also given good readings. According to Lazear et al. (1958), 0.03% methylene blue is preferable if it is intended to take black-and-white photographs.

Cups

Using as a punch a piece of glass tubing 7 mm in internal diameter and 8 mm in external diameter, remove seven small vertical cylinders of the solidified medium so as to leave six cup-shaped holes spaced 5 mm apart around the circumference of a circle 14 mm in radius, with the seventh hole at the centre. These dimensions were fixed after having determined the distance between the antibody source and the antigen source giving the best differentiation of the precipitation lines. A drop of the molten medium is then pipetted into each cup so as to ensure that the bottom is sealed. This is necessary to stop any leakage of serum, which would mix with the antigen and prevent the formation of precipitation lines.

Antigen

For the identification of sera, the authors used as antigen either brains of dogs infected with a highly virulent strain of street virus or brains of young rabbits inoculated with the 1955th passage of the Pasteur fixed virus strain.

Antibody

The authors used either purified horse serum produced by the Institut Pasteur or crude serum from donkeys immunized by a series of injections of HEP Flury virus followed by a series of injections of LEP Flury virus, immunity being maintained by monthly injections of 24 ml of a 33% suspension of chicken embryo infected with an LEP Flury strain.

Test procedure

Two techniques can be used. The first consists in placing rabid brain tissue and normal brain tissue in alternative peripheral cups and running antirabies serum into the central cup ; the normal tissue serves as a control. The second technique is the reverse of the first, i.e., the peripheral cups are filled alternately with immune serum and normal serum, while rabid brain tissue is placed in the central cup.

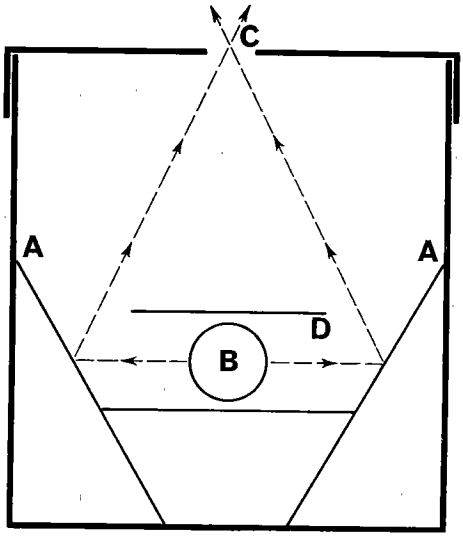
Reading the results

The Petri dishes are placed in an incubator at 37°C and the cups refilled with serum at intervals of about 12 hours, or as often as necessary to prevent them becoming empty. Readings are taken after 24, 48 and 72 hours, depending on the time of appearance of the precipitation lines. They are facilitated by the use of dark-ground illumination. This may be provided by means of a simplified version of the device described by Lazear et al. (1958) : a plywood box with detachable lid is lined with silver paper and two plane mirrors are so arranged inside that light from a 100-watt lamp placed midway between them is reflected through a circular hole, 8 cm in diameter, cut in the lid. A screen covered with black paper placed between the lamp and the hole prevents the direct light from reaching the eye, so that the readings are made against a dark ground (see Fig. 1).

Optimum distance between antigen and antibody cups

This should be determined using a known antigen, which is placed in two divergent rows of cups, while a central row, containing the same number of cups as each of the other two rows, is filled with immune serum.

FIG. 1. APPARATUS FOR DARK-GROUND ILLUMINATION



Modified from Lazear et al. (1958)

A. Plane mirrors. B. 100-watt lamp.
C. 8-cm hole in detachable lid of silver-paper lined box
D. Screen covered with black paper.

According to experiments made by Villemot & Provost, the precipitation lines are most easily and rapidly observed using cups 7 mm in diameter placed 7 mm apart.

Interpretation of results

It is necessary to make sure that suspensions of normal animal brain do not give a precipitate when tested against the serum. If normal rabbit brains are tested against serum produced by immunization with rabbit brain, a precipitation line appears after 48 hours and a second line, fainter than the first and nearer to the antigen cup, appears after 72 hours. These lines are due to the presence in the serum of anti-rabbit-brain antigen ; if the antigen is first removed by mixing the serum in a mechanical shaker with ground normal rabbit brain and then recovering the serum by centrifugation, the lines do not appear.

If the brain of a rabid dog is used, two lines appear after 48 hours. These curve very slightly away from the antigen cups. They are attributed to two antigen fractions, one with a molecular weight approximately equal to that of the antibody, the other with a molecular weight slightly higher.

Microprecipitation on slides

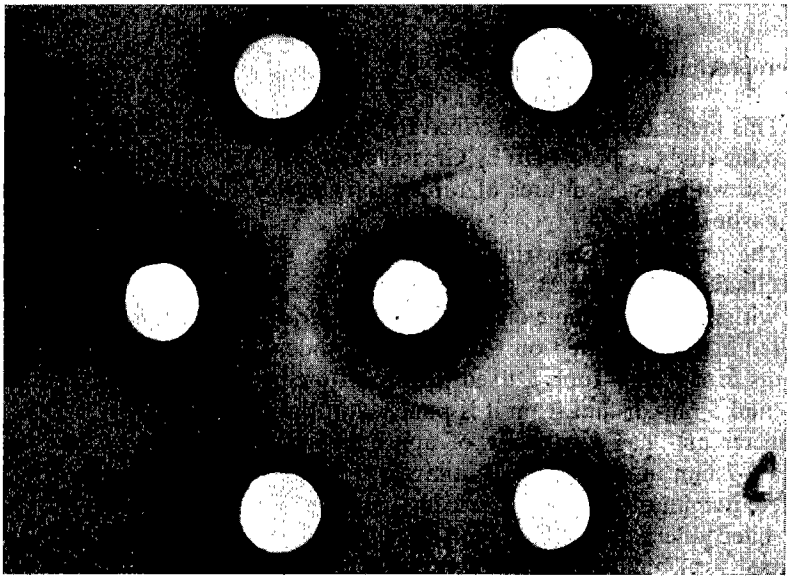
This method was first described by Scheidegger in 1955 and later adapted to the study of poliovirus by Grasset et al. (1958). A modification suitable for use with tissue cultures of rabies antigen was developed by Grasset & Atanasiu (1961).

The method of preparing the microscope slides is similar to that used by Grasset et al. A 2% solution of agar is diluted immediately before use with an equal volume of barbiturate buffer of pH 8.2. Next, 3 ml of the melted agar are poured on to histological slides and allowed to solidify. By means of a special tool, a fixed pattern of 6 cups disposed about a central cup and 5 mm distant from it is punched in the agar. Each cup is 2 mm in diameter and has a capacity of approximately 0.004 ml (in some cases, cups 5-7 mm in diameter with a larger capacity have been used). Concentrated immune serum is placed in the central cup. The peripheral cups are filled alternately either with tissue culture of the rabies antigen and control tissue culture or with concentrated brain suspensions from animals suspected of rabies and from control animals ; the samples should be taken from Ammon's horn, the hippocampal gyrus, or the basal optic ganglion (see chapter 4, page 49). The slides are then left at laboratory temperature in an air-tight vessel containing an atmosphere saturated with water vapour. The precipitation lines begin to appear after 12 hours. After 40-72 hours, the slides are dried and stained with amidoschwartz solution.

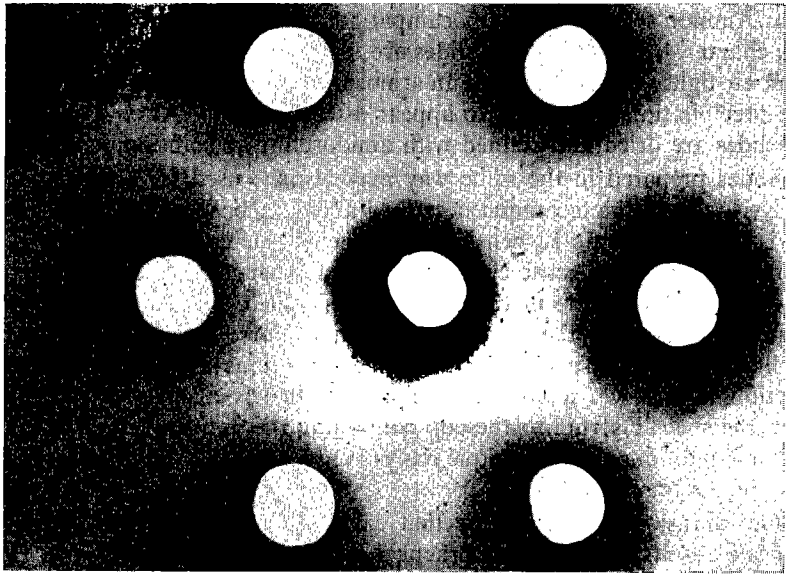
Slides prepared in the same way may also be used for electrophoretic analysis. As in the technique described above, a barbiturate buffer of pH 8.2 is used ; the brain suspension or antigen tissue culture is placed in the central cup and an electric current is passed through the gel. Two lateral grooves are then made in the agar, one of which is filled with antirabies immune serum and the other with normal horse serum. Precipitation is allowed to take place under the same conditions as before. When the reaction is positive, a double precipitation line is again seen, extending from the specific serum to the cup containing the antigen (Fig. 2). Under certain conditions, this technique has proved sensitive for quite low titres of virus in the brain (10^{-2}).

Gel diffusion is a technique that calls for careful execution but gives accurate results. What is its practical value ? Villemot & Provost (1958 a, b) have employed it on a routine basis for the examination of brains sent to their laboratory. Their material comprised 44 brains from dogs, 16 from cats, 2 from monkeys and 2 from human patients, all of whom were suspected of rabies. The test gave a positive result with 24 canine brains and one human brain. These findings were in 100% agreement with the results of animal inoculation experiments, but they were obtained in 48 hours whereas animal experiments take much longer.

FIG. 2. MICROPRECIPITATION ON SLIDES



A



B

Reproduced, by permission, from Grasset & Atanasiu (1961)

A. Double precipitation line resulting from reaction of rabies antigen with specific antirabies serum. The thicker, principal line is situated near the central cup containing the rabies antigen. The thinner, secondary line is situated half-way between the central cup and the three cups on the left containing the antirabies serum. No line is visible between the central cup and the three cups on the right, which contain normal horse serum.

B. Control slide. No lines are visible on either side of the central cup containing the control antigen (tissue-culture preparation). The three cups on the left contain antirabies serum, the three on the right normal horse serum.

Magnification ×4.

Atanasiu et al. (1963) used the technique of Grasset & Atanasiu (1961) to identify rabies virus cultured on $BHK_{21}C_{13}$ cells in the presence of hyper-immune horse serum from the Pasteur Institute. The results were excellent.

The gel-diffusion technique is thus both specific and sensitive. However, it has its limitations. Thiéry (1960) has made a comparison between the method of Villemot & Provost and the classical methods for the routine identification of rabies virus. He concludes that when brain tissue is examined by the gel-diffusion method, the result will be positive if sufficient rabies antigen is present; when serum is examined, a positive result is obtained if there is an increase in the α_2 globulins, an increase that is normally associated with rabies but varies in degree from one animal to another. From this it follows that, while a positive result is unequivocal evidence of rabies, a negative result may be due to the absence from the antigen or the serum of the specific fractions characteristic of rabies or to a disproportion between the quantities of antigen and antibody entering into the reaction. Grasset & Atanasiu likewise found that the immuno-precipitation reaction gave consistent results only if antigen and antibody were present in suitable proportions; they obtained negative results not only with tissue cultures or brain tissues having a low virus titre although giving a positive inoculation test, but also with sera of low neutralizing power.

It must therefore be concluded that although the gel-diffusion technique is accurate and sensitive it cannot at present be considered entirely reliable. A positive result permits a rapid diagnosis of rabies or the presence of rabies antibody. On the other hand, a negative result does not necessarily exclude rabies or the presence of rabies antibody. Further research is needed to determine the precise applications of the method to the diagnosis of rabies.

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PART II

METHODS OF VACCINE PRODUCTION

CHAPTER 9

GENERAL CONSIDERATIONS IN VACCINE PRODUCTION

KARL HABEL [1]

The principles involved in the production of satisfactory rabies vaccines are no different from those considered generally applicable to any virus vaccine. The obviously important considerations are safety, potency, ease of production, and stability even after prolonged storage. To date, no one type of rabies vaccine has proved superior in all these characteristics, and there is therefore a choice among several products each of which has some special advantage.

In general, with increasing emphasis on production procedures aimed at increasing the safety of the product there has been a tendency for potencies to drop. This is seen in the duck-embryo vaccine where the small amount of nervous tissue greatly reduces the hazard of post-vaccinal complications of the central nervous system, but the immunogenic potency is usually somewhat less than that of the average brain-tissue vaccine. Chemical methods are available for removing the allergic encephalitic factor from brain-tissue vaccines, but the cost would be prohibitive.

Preservation of potency on prolonged storage can be achieved by lyophilization but formerly this was possible only with vaccines inactivated by a virucidal agent that could be removed (ultraviolet irradiation, beta-propiolactone). Now, however, the advantages of this technique have been extended to the phenolized vaccines also.

Live virus vaccines, using virus grown in the chicken embryo, have proved vastly superior to inactivated vaccines in veterinary use. These vaccines contain rabies virus strains attenuated by avian embryo passage to the point where they do not produce clinical infection on inoculation of the proper species, yet are still capable of multiplying in the vaccinated host. However, both these properties vary in different species. Although attenuated for dogs, the LEP Flury vaccine cannot be used in cattle for here it is still virulent, while the HEP Flury vaccine is avirulent for this species. On the other hand, the chicken-embryo live virus vaccines have limited advantages in man, for here the attenuated vaccine virus fails to multiply. The fact that virus strains can vary in their virulence and their ability to immunize different animal species, emphasizes the need to use

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well-established strains, such as the Flury strains, for routine live virus vaccine production. There is no evidence of any advantage of local strains for this purpose, and an extensive background of field experience is difficult to obtain with newly developed attenuated strains of virus. In the case of live virus vaccines, the great importance of obtaining the necessary infective virus titre in production lots, and of the lyophilization process to preserve this titre, is obvious. If at the time of field use there is not sufficient viable virus to initiate the inapparent infection necessary for immunization of the vaccinated animal, these vaccines will not protect.

In general, rabies virus is a relatively poor antigen in inactivated vaccines. This is the reason that the presence in the final vaccine of a large amount of the antigen must be ensured by using a virus source of high titre for inactivated vaccine production. Materials having an infectivity titre of less than 10^5 LD_{50} per 0.03 ml (for the mouse inoculated intracerebrally) will not make an inactivated vaccine of satisfactory potency. This low antigenicity is the reason for large vaccine doses, repeated inoculations, and the difficulty in retaining potency after attempts at purification. Yet in spite of this, the efficiency of booster doses of rabies vaccine has been definitely established and basic immunity may persist for many years.

There are a number of types of inactivated virus vaccine that appear satisfactory. In general, any method is acceptable which gives a safe and quantitatively proven potent vaccine. However, the ideal vaccine would be prepared from virus source material containing no proteins foreign to humans, would be easily and inexpensively produced in large volumes with uniformly high titres, and would be inactivated by a method that consistently retained high immunizing potencies ; furthermore, it would be capable of long-term storage without a decrease in potency and would cause no reactions on repeated inoculation of patients. Since none of the presently available vaccines can meet all these requirements, research for vaccine improvement continues.

FERMI-TYPE VACCINE

PIERRE LÉPINE [1]

Formula of Vaccine

The vaccine consists of a 5% aqueous suspension of brain tissue from sheep inoculated with fixed rabies virus, inactivated with phenol. The initial concentration of phenol is 1%, but this may be reduced to 0.5% in the final vaccine. Although Fermi-type vaccine is usually classed as an inactivated vaccine, it contains a precisely determined quantity of active virus in addition to the virus inactivated by the phenol. It is thus a mixed attenuated-inactivated type of vaccine.

Rabies Strain Employed

The strain employed is the Pasteur Institute fixed virus (Louis Pasteur strain) isolated on 19 November 1882, which has been maintained solely in the rabbit since that date. On its 90th passage this strain was used for the first human immunization (6 July 1885) and since then it has been constantly employed at the Pasteur Institute in Paris for immunization purposes. The behaviour and characteristics of the strain are periodically examined and have formed the subject of much research.

Since 1 April 1952 the Pasteur strain, which was formerly subjected to continuous passage in the rabbit, has been passed regularly once a month. For these passages of the strain, at least two rabbits with a minimum weight of 2 kg each are inoculated intracerebrally with 0.25 ml of a 1 : 10 suspension of brain tissue prepared from the preceding passage (the brain is kept in glycerol at $+5^{\circ}C$). The rabbits, which become paralysed on the sixth day, are sacrificed by bleeding on the seventh day. The brains, which are extracted under sterile conditions, are subjected to bacteriological sterility tests, histological examination for the detection of typical fixed-virus lesions (see Plate I, F, G & H, facing page 48) and titration of the virus by inoculation of a dilute suspension of fresh virus into rabbits.

Immediately after removal, the brain is placed in pure, sterile glycerol and dispensed in 25-ml portions into wide-necked bottles. Labels indicating the number of the rabbit and of the passage are placed on all bottles,

[1] Chief, Virus Division, Pasteur Institute, Paris.

which are kept in the refrigerator at between +2°C and +5°C for at least 48 hours. The glycerolated brains are then stored at a temperature of −20°C, providing a stock of virus for the preparation of the inoculum.

By 1 June, 1964, the Pasteur strain had undergone 2045 passages since its isolation.

Preparation of Seed Virus

For inoculating the sheep the last passage of the strain is used, i.e., a brain from the regular series of passages, kept in glycerol in the refrigerator for more than a week but less than a month. A fragment of brain is removed, rinsed under sterile conditions with sterile distilled water, and weighed. It is then ground up in a chilled mortar so as to obtain a 1 : 10 emulsion of brain (by weight) in physiological salt solution. This constitutes the seed suspension. Each sheep is inoculated intracerebrally with 0.5 ml of the suspension at one point of the brain, using the technique described below.

Preparation of Sheep

In principle, the vaccine is prepared using young sheep. However, goats are equally satisfactory if more readily available; kids or young adults should always be selected and the inoculation made intracerebrally. At the Pasteur Institute, young sheep in good health and having an average weight of 42 kg are invariably used. The brains of these animals weigh

FIG. 1. TABLE FOR RESTRAINING SHEEP FOR INTRACEREBRAL INOCULATION

FIG. 2. SITE AND TECHNIQUE FOR TREPHINING OF SHEEP

about 80 g. The animals are shorn and kept under close veterinary supervision in individual pens for three weeks. All those whose condition does not appear to be completely normal are eliminated.

On the day before inoculation the head of the sheep is shaved and the skin then disinfected by vigorous brushing with cresol solution, followed by painting with tincture of iodine. This last procedure, which is carried out in the morning and evening, is repeated on the morning of the next day. At the time of inoculation the shaven skull of the animal has thus been disinfected once with cresol and three times with tincture of iodine.

The animal is taken to the inoculation laboratory and immobilized on a special table (see Fig. 1). The skin is held taut by an assistant, and trephining performed, without a previous incision, using a trephining drill with a point 13 mm long and 2 mm in diameter (see Fig. 2). The point of the instrument is directed towards the lateral-external region of the left hemisphere of the brain (lateral face of the frontal lobe). As mentioned above, each animal receives 0.5 ml of the seed-virus suspension in a single injection.

Several sheep are always inoculated during the same session, the usual number being eight sheep per session. The sheep show the first symptoms of rabies seven to eight days later, and all are sacrificed on the ninth day when they are already unable to stand but are still alive. The animals are

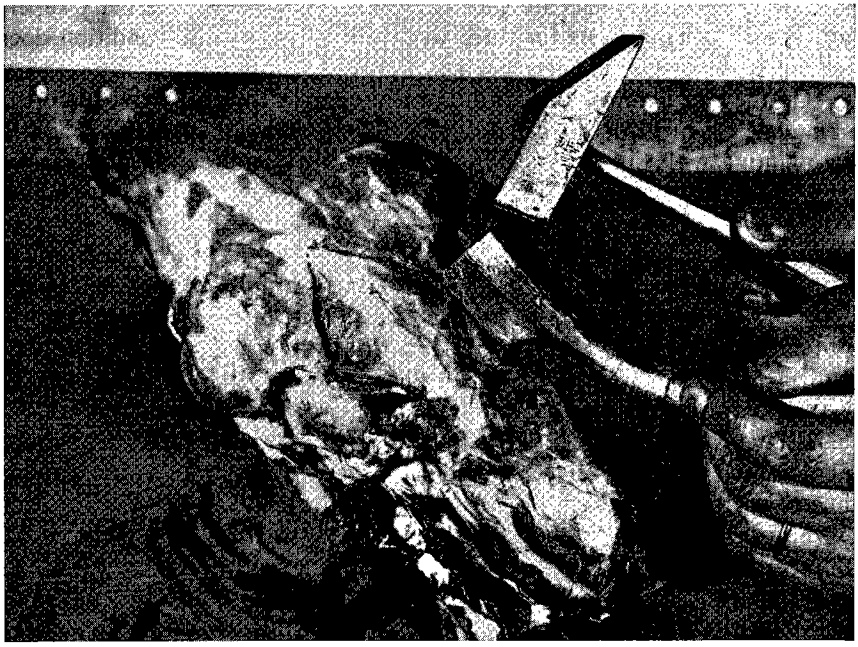
killed by total exsanguination from the carotid artery, and the brain is immediately removed.

As a general rule, whatever the strain employed the inoculated animals are killed as soon as the usual prodromal symptoms, such as rise in body temperature, mydriasis, trembling and sluggishness, are followed by definite signs of paralysis, i.e., at the onset of clinical evidence of encephalitis. In some institutes it is the practice to wait until the terminal stage of generalized paralysis has been reached before killing the animals, but this is inadvisable as it not only yields brain tissue of lower virus titre, but also considerably increases the chances of bacterial contamination from the septicaemia that develops during the terminal stage.

Removal of the Brain

Immediately after death the head of the animal is separated by cutting through the middle of the neck, the skin is completely removed, and the head is placed on a tray. The whole bared surface of the skull is treated with iodized ethanol and carefully flamed with a bunsen burner. Using an autopsy hammer and a chisel, a lozenge-shaped opening is then made in the skull, parallel to the longitudinal axis of the head (see Fig. 3).

FIG. 3. TECHNIQUE FOR OPENING SKULL



This opening extends lengthwise from a line joining the orbits to the crown of the head and, in width, from one edge to the other of the skull cap. After incision of the meninges and cutting through the base of the medulla (see Fig. 4), the brain is extracted and placed in a sterile container (see Fig. 5). Samples are then removed for checking the sterility of the various parts of the brain. Each brain is placed in a sterile glass container (Pyrex-glass preserving-jar with airtight cover) and frozen at −25°C while awaiting the results of the sterility tests and titration of its virus content.

FIG. 4. APPEARANCE OF SKULL AFTER OPENING AND LIFTING BACK OF CALVARIA

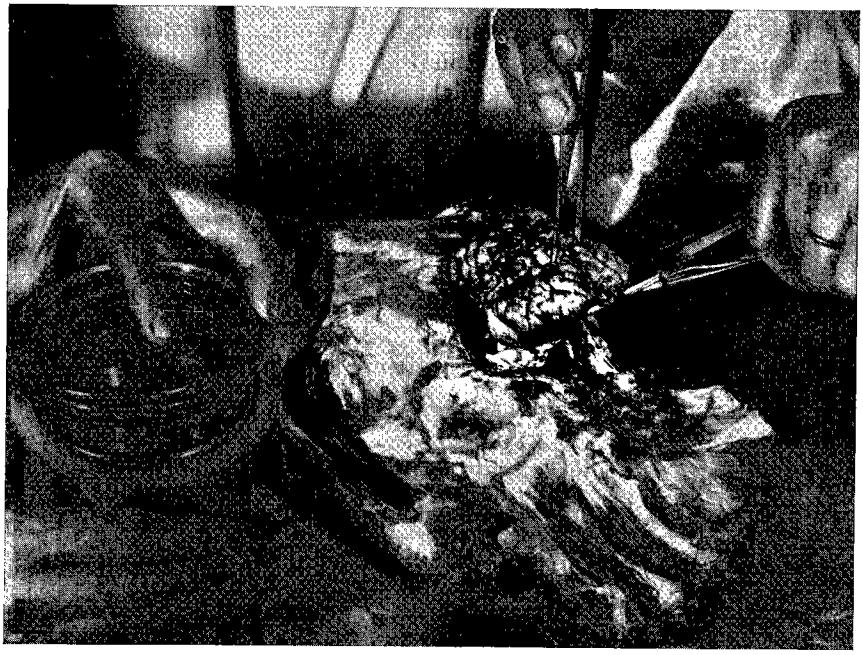
Finally, a thorough post-mortem examination of the animal is made; no evidence of disease other than rabies should be found. The results are noted down and kept with the report on the preparation of the vaccine.

Sterility and Virulence Tests

Fragments from the cortex and from the brain stem are removed with sterile precautions and seeded in enriched nutrient broth, in ordinary tubes and in tubes of the Hall type, for aerobic and anaerobic tests. The cultures are kept for eight days at 37°C. At the same time, a control titration of virulence is carried out as described previously by intracerebral

inoculation of rabbits with increasing dilutions of the brain-tissue suspension.

FIG. 5. EXTRACTION OF SHEEP BRAIN



Left—sterile Pyrex-glass jar in which brain is kept at low temperature

Preparation of Vaccine

General principles

As explained above, after the brains have been removed from the rabid sheep, they should be stored dry in sterile containers at as low a temperature as possible, preferably not above —25°C. They should not be kept for more than two months.

Pure, white, crystalline phenol is used for the preparation of the vaccine. As soon as it is received in the laboratory, it is dispensed into ampoules, which are kept sealed. Any batches of phenol showing a slight reddish coloration (indicating some degree of oxidation) should be rejected.

The physiological saline used for dilution of the vaccine should have a pH above 7—preferably 7.6—so that the final pH will be 7.4. Physiological saline of pH 7.6 is obtained by dissolving 8.5 g of NaCl in 1000 ml of twice distilled water (or water demineralized by an ion-exchange process) and adding 15 ml of an M/15 solution of KH_2PO_4 and 85 ml of an M/15 solution of $Na_2HPO_4.12H_2O$.

Technique

The brains are taken from the refrigerator in their special containers and rapidly thawed in a water-bath at 37°C. The brains are then weighed separately and ground up in a Waring blendor, cooled by a jacket filled with crushed ice. Two brains at a time (about 160 g of brain) are placed in the apparatus. Grinding is performed without diluent, with the machine running at full speed for a period of four minutes. A small amount of sterile physiological salt solution is then added. After having allowed the blendor to run for three minutes to ensure that the mixture is homogeneous, samples are taken for the control culture and the final dilution is made in large Pyrex containers holding five litres.

Sterile physiological saline, prepared as described above, is phenolized as required by the addition of sufficient pure phenol to give a 5% solution. This is then diluted immediately before use in the proportion necessary to give the required final concentration of vaccine.

The vaccine mixture is agitated and then left for 24 hours in an oven at 22°C. In areas where the ambient temperature is usually above 22°C, the use of a constant-temperature cabinet capable of being held at this temperature is strongly recommended. Inactivation should never be carried out at a temperature below 22°C.

During inactivation, the concentration of phenol should be 1 %. Some laboratories may prefer to dilute the vaccine after inactivation. For example, if a vaccine containing 10% of brain tissue and 1% of phenol has been inactivated for 24 hours at 22°C and then kept for days at +4°C, it may be subsequently diluted with an equal volume of physiological saline to give a brain-tissue concentration of 5% and a phenol concentration of 0.5%. At all events, the concentration of brain tissue in the final vaccine should be 5%.

The proportions of diluent and phenolized saline are calculated according to the weight of ground brain tissue. Thus, to obtain a vaccine having a final concentration of phenol of 1%, the following proportions are used :

brain tissue	1 g
sterile physiological saline	16 ml
5% phenol in physiological saline	4 ml

Alternatively, a vaccine having a final concentration of phenol of 0.5% is obtained using the following proportions :

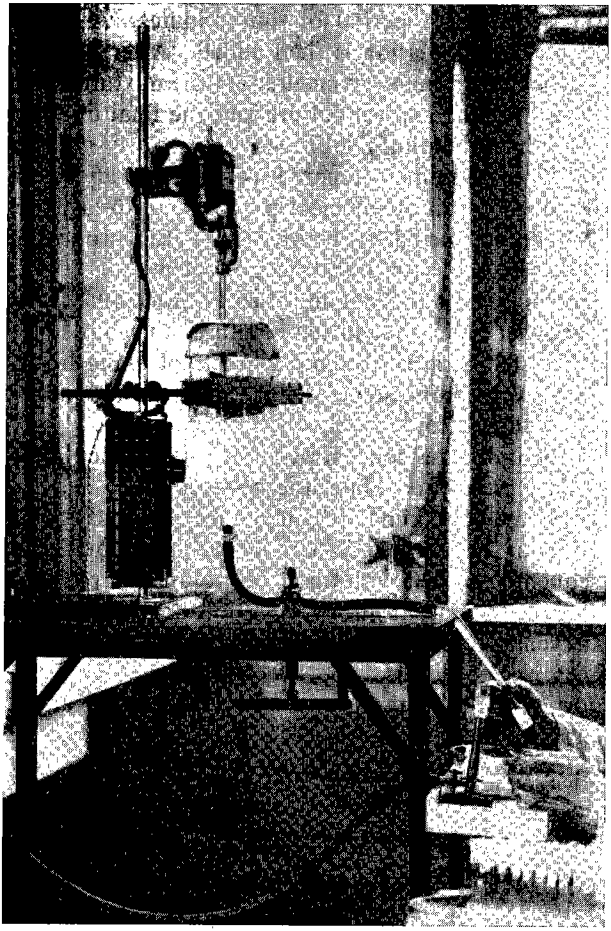
brain tissue	2 g
sterile physiological saline	16 ml
5% phenol in physiological saline	4 ml

and diluting after inactivation with :

physiological saline	20 ml

On removal from the oven, the vaccine is immediately dispensed into 5-ml ampoules. It is essential that the vaccine should be homogeneous to ensure that exactly the same quantity is dispensed into each ampoule. The vaccine is therefore kept stirred during the filling process, using an electri-

FIG. 6. FILLING AMPOULES WITH ANTIRABIES VACCINE



Note the turbo-stirrer which ensures even mixing of the vaccine suspension during filling

cally driven turbo-stirrer with a detachable stainless steel shaft, approximately 35 cm in length, to which are connected a stainless steel adapter and turbine (Fig. 6). Before use, the shaft, adapter and turbine are detached from the motor and sterilized. The turbo-stirrer has the advantages over

ordinary stirrers that it does not cause frothing and results in a much more homogeneous emulsion of microscopic particles. This makes it possible to fill into ampoules an unlimited quantity of vaccine with the certainty that it will be rigorously homogeneous and consequently that each ampoule will contain the correct dose.

The filled ampoules are kept for at least a week at between +2°C and +5°C before being sent on to the packing department. During this storage period, which is necessary to complete the inactivation of the virus, the final control of the vaccine is carried out. Storage of the vaccine at the temperature range mentioned above is indispensable and should be considered an integral part of the method of preparation.

Tests on the Final Vaccine

Before being issued for use, the vaccine undergoes the following tests :

(1) Sterility tests, as described above.

(2) Test for inactivation and residual virulence by inoculation of rabbits (see Annex 1).

(3) Potency test. Either the normal Habel test (see chapter 16, page 140) or the modified Habel test (see chapter 17, page 144) may be used, provided that the vaccine is being manufactured in large quantities on a routine basis. For small quantities of vaccine, manufactured at irregular intervals, the normal (complete) Habel test must always be used. The modified test permits a considerable economy in the number of mice required, but the results should be checked periodically by carrying out the complete test.

(4) Determination of the pH of the vaccine and titration of the free phenol (see Annex 2).

Expiry Date

The expiry date is five months from the date of issue, provided that the vaccine is kept at a temperature between 2°C and 5°C throughout this time. If low-temperature storage cannot be guaranteed, the expiry date is reduced to 2-3 months from the date of issue, depending on the climate.

Freeze-Dried Antirabies Vaccine

The following technique has been adopted at the Pasteur Institute for the preparation of freeze-dried antirabies vaccine of the Fermi type.

Sheep are infected with the Pasteur fixed strain of rabies virus, now at

its 2051st passage. The sheep brains are ground up with twice their weight of normal saline. To the ground-up suspension is then added sufficient diluent to give a final product containing: [1]

brain tissue	10%
phenol	1%
sucrose	15%
gelatin	0.5%
sodium chloride	0.85%

The resulting vaccine is kept for 24 hours in the incubator at 22°C. It is then dispensed in 2.5-ml quantities into 20-ml bottles of the penicillin type and centrifuged. The residue is lyophilized by freezing at −40°C and desiccating for 20 hours, allowing the temperature to rise slowly to a maximum of 23-24°C at the end of the process ("Usifroid" apparatus). The bottles are sealed under nitrogen.

When required for use, the vaccine is reconstituted by adding 5 ml of sterile, twice-distilled water to each bottle. This gives a volume of 5 ml of vaccine containing 5% of brain tissue.

Physico-chemical tests on the vaccine

All lots prepared are tested for their pH and phenol content. After reconstitution, the pH of the vaccine varies between 5.4 and 4.8 and its phenol content between 0.22 % and 0.40%.

Virulence test

The reconstituted vaccine is not completely avirulent when inoculated intracerebrally into mice, but the batches prepared so far have been found to be considerably less virulent than the liquid Fermi-type vaccine (see Annex 1).

Potency

The potency of the freeze-dried vaccine as determined by the Habel test is very satisfactory. The protective titre (log "LD_{50} of protection") determined on 5 successive lots immediately after lyophilization was between 3.6 and 4.6; after incubation for 7 days at 37°C, values ranging from 4.8 to 5.6 were found.

[1] The chemicals used in the preparation of the vaccine must be of analytical grade.

Annex 1

TEST FOR RESIDUAL VIRULENCE OF THE FINAL VACCINE

The method of inactivation described above results in a vaccine that still possesses a certain residual virulence. Each final lot of vaccine must be tested in mice to determine its residual virulence. This test should be performed 9 days after the addition of phenol ; in cases of extreme urgency, it may be performed sooner, but not less than two days after the addition of phenol.

A sample of the vaccine to be tested is submitted to low-speed centrifugation and the supernatant diluted 10^{-1}, 10^{-2} and 10^{-3} with physiological saline of pH 7. Each dilution is administered by intracerebral inoculation to a group of at least 5 (preferably 10) mice, each animal receiving 0.03 ml. The three groups of mice are kept under observation for 15 days and a record is made of the number of paralysed animals that die from the 3rd day onwards.

The residual virulence of the vaccine should be such that

(*a*) all animals die with the 10^{-1} dilution ;

(*b*) the mortality with the 10^{-2} dilution is more or less constant (not more than 50%) ;

(*c*) no deaths occur with the 10^{-3} dilution ;

(*d*) the virus titre, as calculated by the Reed & Muench method, should not be greater than $10^{-2.7}$.

Annex 2

DETERMINATION OF THE PHENOL CONTENT OF THE ANTIRABIES VACCINE

1. Theis and Benedict Method

Principle

The method described makes use of the orange coloration given by phenol with diazotized paranitraniline in an alkaline medium. This coloration, due to the formation of an azo compound, is suitable for photometric assay.

Reagents

1% Gum arabic solution
50% Sodium acetate solution
20% Sodium carbonate solution
Hydrochloric acid
40% Sodium hydroxide
Diazotized paranitraniline solution, prepared as required from the following two reagents:

A	paranitraniline	1.5 g
	pure hydrochloric acid	40 ml
	distilled water	to 500 ml
B	sodium nitrite	1 g
	distilled water	to 10 ml

Immediately before use, 25 ml of reagent A are mixed with 0.75 ml of reagent B, with cooling. The mixture does not keep for more than 24 hours.

Apparatus

1-ml pipette
2-ml pipette
20-ml pipette
Test tubes
50-ml standard flasks
Electrophotometer

Procedure

Shake the ampoule well before opening and then transfer the contents to a test tube. Using a 1-ml pipette, transfer 1 ml of the vaccine from the test tube to a 50-ml standard flask containing 30-40 ml of distilled water (shake the test tube before removing the sample). Make up to 50 ml with distilled water, giving a 1 : 50 dilution.

Shake the flask well to render the solution homogeneous. Using a 2-ml pipette, remove 2 ml of the 1 : 50 dilution and transfer to a second 50-ml standard flask. Add 20 ml of distilled water from a pipette and shake. Then add 1 ml of 1% gum arabic solution, 1 ml of 50% sodium acetate solution and 1 ml of *cooled* diazotized paranitraniline solution (mixture of reagents A and B). Shake and allow to stand for 1 minute. Then add 2 ml of 20% sodium carbonate solution. Shake, make up to 50 ml with distilled water and shake again.

Fill an electrophotometer cell with the solution and measure the colour in the electrophotometer, using a green filter. The photometer should be calibrated beforehand with a series of phenol solutions of known concentrations, treated in the same way as the sample of vaccine under test. It is convenient to plot a calibration curve, so that the phenol concentrations can be read off directly.

2. Koppeschaar Method

Principle

If an aqueous solution of phenol is treated with an excess of bromine, the phenol is converted quantitatively into tribromphenol; if an excess of potassium iodide is then added, iodine is liberated by the excess bromine and the quantity can be determined by titration. This enables the amount of excess bromine to be calculated and, hence, the amount of phenol originally present.

Procedure

Measure out 5 ml [1] of vaccine into a 250-ml conical flask. Add approximately 120 ml of distilled water followed by exactly 25 ml of 0.2N potassium bromate solution and 25 ml of N potassium bromide solution. Then add 5 ml of pure concentrated hydrochloric acid (specific gravity 1.19) with shaking. Allow to stand for 15 minutes, add 2 g of potassium iodide, shake and allow to stand for another 2 minutes.

Titrate the liberated iodine against 0.1N sodium hyposulfite solution. If the volume of solution needed is n ml, then the number of grams of phenol present in 100 ml of vaccine is given by the formula:

$$(50-n) \times 0.0313.$$

It is important that the solutions of N potassium bromate and 0.1N sodium hyposulfite should be accurately standardized.

[1] This quantity was selected on the basis of a 1% phenol content.

SEMPLE-TYPE VACCINE

EDWARD B. SELIGMANN, jr, Ph.D.[1]

The following procedure is recommended to meet the requirements of the US National Institutes of Health [2] and is based on successful results obtained by vaccine manufacturers in the USA. While rabbits are used exclusively in the USA for the manufacture of brain-tissue vaccine for administration to man, sheep and goats may also be used.

Formula of Vaccine

Each individual human injection of finished killed virus vaccine should contain not less than 2.0 ml of a 5% suspension of inactivated rabies fixed virus in phosphate-buffered saline (pH 7.0), or its equivalent in another concentration. The finished vaccine should contain not more than 0.25% of phenol for vaccines containing 10% or less of brain tissue or not more than 0.4% of phenol for vaccines containing more than 10% of brain tissue, as calculated from the amount originally added. In addition, either 0.008% of phenylmercuric borate or 0.01% of thiomersal should be added.

Preparation of Seed Virus

Virus strain

The production strain used in the USA is a strain of fixed rabies virus selected on the basis of the high antigenicity of vaccines produced from it when challenged with a variety of other strains. This strain has been designated the PV strain.[3]

Maintenance of virus strain

Virus passage of the seed strain used for vaccine production is always through rabbit brain. A 10% suspension of infected rabbit brain in sterile distilled water is maintained in either the frozen or freeze-dried state.

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[1] Chief, Reference Standards Section, Division of Biologics Standards, National Institutes of Health, Bethesda, Md., USA.

[2] United States National Institutes of Health (1953) *Minimum Requirements : Rabies Vaccine*, 3rd rev., Bethesda, Md.

[3] Available to national laboratories upon request from the World Health Organization, Geneva, Switzerland.

Intracerebral inoculation of 0.25 ml of a 10^{-3} dilution of this preparation should produce paralysis in rabbits within 5 to 7 days after injection. Seed virus may be made as a 10% suspension of infected brains in sterile distilled water distributed into ampoules, glass-sealed and quick-frozen. The ampoules are preferably stored in a dry-ice [1] chest (—70°C), but in any case at a temperature lower than —15°C. The storage period may be as long as full potency is maintained as shown by mouse titration. The seed virus suspension should be of such activity as to kill all mice inoculated intracerebrally with 0.03-ml quantities in a dilution of not less than 10^{-6}.

The specificity of the virus strain is tested periodically by titration in mice against specific antirabies hyperimmune serum which has proved protective against street virus (see chapter 22).

Production of seed-virus 10% suspension

Rabies-infected rabbit brains are harvested from rabbits that have been inoculated intracerebrally with rabies virus and that have shown typical symptoms of rabies. The brains are harvested after the animals have developed complete paralysis but just prior to death. The brains are quick-frozen immediately and stored in a dry-ice chest until needed. If processed without freezing, all steps should be done as rapidly as possible, keeping the temperature as low as practicable. The brains are weighed and emulsified in a small volume of cold sterile distilled water. Emulsification is effected in a Waring blendor of appropriate size. The frozen brains should preferably be minced by the blendor blades and then a portion of the cold distilled water added for emulsification. Blending should be done in such a way as to minimize the rise in temperature. A total time of 2-3 minutes is usually all that is necessary. When the brains have been emulsified to a smooth paste, the rest of the cold distilled water can be added to yield a final 10% suspension (w/v) and blended briefly. The suspension is placed in appropriate volumes in ampoules, glass-sealed, and quick-frozen in dry-ice and alcohol. The ampoules are preferably stored at dry-ice temperature (—70°C), but in any case at a temperature lower than —15°C.

Sterility

The seed lot should be free from bacterial and fungal contaminants as indicated by an appropriate test (see page 115).

Virus titration

At least three intracerebral mouse titrations of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions are performed. The test period is 14 days, and all mice

[1] Solid carbon dioxide.

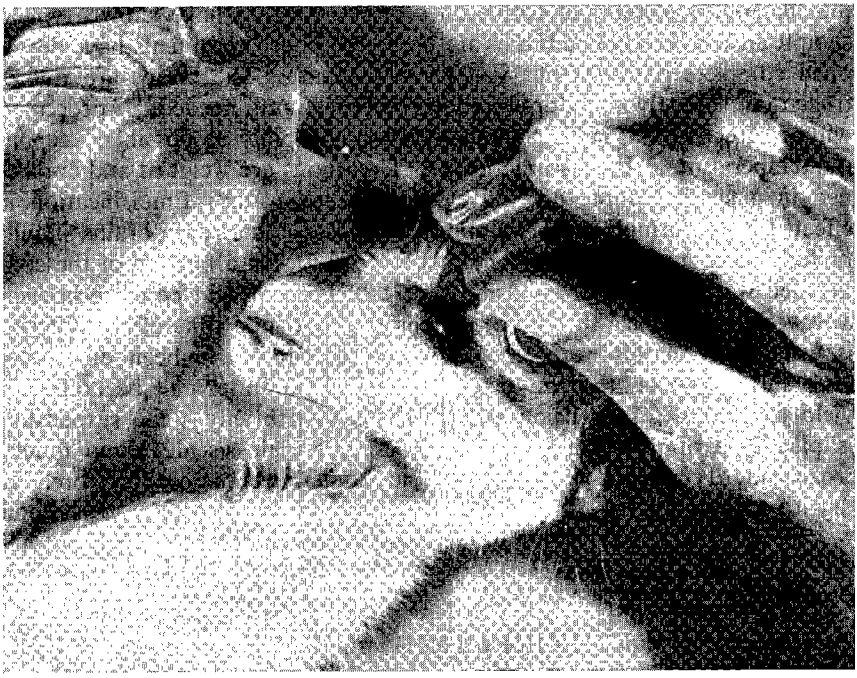
receiving the 10^{-5} dilution should die. If the virus has been properly prepared it can be expected that all mice receiving the 10^{-6} dilution will also die.

Inoculation and Harvest

Preparation of inoculum and inoculation technique

From the 10% suspension of seed virus a final 10^{-3} solution of virus is made in 2% normal horse serum in distilled water. It is not necessary to centrifuge this preparation. A fresh dilution is made for each day's inoculation.

INTRACEREBRAL INOCULATION TECHNIQUE



Healthy rabbits are firmly secured or lightly etherized and 0.25 ml of the inoculum is introduced by a sterile 2-ml syringe using a 20-gauge $^3/_8$-inch needle (0.65 mm × 1 cm) through the skull approximately halfway between the outer canthus of the eye and the ear (see figure). A stainless-steel awl or a punch may be used to make the puncture in large rabbits.

Progress of disease in rabbits

Rabbits show marked signs of paralysis by the 4th day or occasionally as late as the 7th day following inoculation. The infected animal should show symptoms of fixed rabies virus paralysis and be moribund before the brain is harvested.

Killing of rabbits

Moribund rabbits that are completely paralysed but still breathing are killed by intravenous injection of approximately 20 ml of air into the marginal vein of the ear. Death occurs in one to two minutes. The brains from freshly killed rabbits only are used for vaccine production ; animals already dead from rabies or other causes are not used.

Autopsy

To guard against the use of unsuitable rabbits, autopsies are performed after brain removal. The brains of rabbits showing the presence of coccidiosis elsewhere in the body are not used because of the chance that coccidiosis rather than rabies may have caused paralysis. A record is made of all autopsy findings.

Brain harvest

The rabbit is washed with 5% phenol or another suitable antiseptic solution. Decapitation is performed as close to the shoulders as possible ; the severed head is fastened securely into a special holder, swabbed with tincture of iodine, and taken into a "sterile room" where actual brain removal is performed.

The skin is slit down the middle of the head and folded back and the exposed top of the skull is washed with tincture of iodine. The top of the skull is then removed with sterile bone-shears, and the brain is transferred with sterile scissors and forceps into a suitable sterile container. A small piece of the brain tissue is removed and tested for bacterial sterility. Brains should be quick-frozen as soon as harvested and stored at —15°C or lower, preferably in a dry-ice chest, until needed for vaccine production. Any brain showing bacterial contamination is discarded.

Manufacture of Vaccine : 40% Suspension

Emulsification

Frozen brains are weighed and placed in a 2-litre conical flask as a 40% suspension in sterile phosphate-buffered saline solution containing

sufficient phenol to give a final concentration of 0.5% (see under *Diluent* below). After the brains have thawed, the flask is shaken by hand until they are well broken up. This partial emulsion is then transferred to a sterile Eppenbach colloidal mill (set at 22-24). Emulsification is completed by one run through the mill. A Waring blendor or other suitable emulsifier can be used ; care should be taken to avoid running the emulsifier long enough to generate appreciable heat. From two to three minutes are usually sufficient for a Waring blendor to emulsify the brains adequately. The container can be surrounded with a jacket of chipped ice and salt to prevent overheating of the contents.

Diluent

(*a*) Phosphate-buffered saline solution : prepare a solution containing 2 parts of M/10 Na_2HPO_4 and 1 part of M/10 KH_2PO_4.

To one part of this solution add four parts of 0.85% NaCl of pH 7.0.

(*b*) To make a 40% tissue-concentration containing 0.5% phenol, add 0.68% of 90% phenol (laboratory reagent grade) to phosphate-buffered saline solution (this allows for the volume of emulsified brain tissue) ; make a 40% weight/volume suspension with the diluent according to the weight of the brains.

Note : After the phenol has been added, great care should be taken not to freeze the brain-tissue suspension, either as the concentrated 40% form or as the final vaccine dilution. *Freezing of phenolized suspensions destroys their antigenicity.*

Virus titre of brain-tissue pool before incubation

A sample of 40% tissue suspension is weighed (because of air content from action of the mill), diluted immediately, and titrated in mice weighing 11-15 g. Dilutions of 10^{-6}, 10^{-7}, and 10^{-8}, representing dilutions of brain-tissue content, are injected intracerebrally and the titre (LD_{50}) should be at least 10^{-6} (see chapter 16, page 142 for calculation).

Incubation

The emulsified vaccine containing 0.5% of phenol is brought to a temperature within the range 20°-30°C in a water-bath for 1 hour and then placed in an incubator within the range 20°-30°C for 72 to 76 hours or until the virus has been inactivated as demonstrated by the innocuity test. The vaccine is agitated mechanically during the entire period in the incubator.

Note : The period in the incubator required for inactivation will vary with different virus strains and methods of emulsification of brain tissue (fineness of particles). The time required should be established in each

laboratory through experimental trials, e.g., intracerebral inoculation of mice with suspension periodically during incubation.

Dilution to 20% vaccine

The 40% vaccine is diluted with an equal volume of the sterile phosphate-buffered saline containing 1 : 5000 thiomersal, so that the finished product contains 20% of tissue, 0.25% of phenol, and 1 : 10000 thiomersal.

Diluted vaccine is passed through several layers of gauze or silk filter for the purpose of removing any unground particles of brain tissue. A convenient batch of vaccine is about 2 litres in volume. It is subjected to the safety and sterility tests described below, and is stored at 4°-6°C.

Sterility test on bulk vaccine

A sterility test on the bulk vaccine is made in fluid thioglycolate medium. A representative sample of at least 10 ml from each container of bulk vaccine should be tested. The sample should be subdivided or placed in a sufficient volume of the test medium to dilute the phenol to a concentration nor greater than 0.008% in each container of sterility test medium. Tests in fluid thioglycolate medium are incubated at 30°-32°C for not less than seven days and examined visually for evidence of growth periodically during this time. In order that growth will not be overlooked because of turbidity, on the 3rd, 4th or 5th day and on the 7th day of incubation, the test containers are mixed and 1 ml from each container is inoculated into additional containers of sterile test medium. All containers are incubated for an additional seven days. Any vaccine showing contamination is retested, using a double volume for the test.

Innocuity test on bulk vaccine

An innocuity test is performed both in rabbits [1] and in mice as a test for the absence of viable virus in the bulk vaccine. This test should be performed on a representative sample taken from each container of bulk vaccine. Not less than two animals of each species are used for each test. Each rabbit is injected intracerebrally with not less than 0.25 ml and each mouse (weight 18-20 g) with 0.03 ml of vaccine containing not less than 5% of brain tissue. All test animals are observed for not less than 14 days during which time each animal must remain free from symptoms of rabies or of other central nervous system disease.

Finished vaccine

After all tests performed on the bulk vaccine have been completed satisfactorily, the vaccine is filled into final containers and labelled.

[1] If another animal is used for production of the vaccine, this test should be carried out in that species and in mice.

Representative samples are taken for the following tests:

Analytical tests

Appropriate tests are performed to determine the total solids, phenol concentration, pH, and thiomersal or other preservative concentration.

General safety test

A safety test for the detection of any inadvertently added toxic substances is made on the undiluted contents of final containers selected at random from each filling of each lot. The parenteral injection of 0.5 ml for mice weighing approximately 20 g each and 5.0 ml for guinea pigs weighing approximately 350 g each should cause neither significant symptoms nor death. At least two animals of each species are used, and the observation period is not less than seven days.

Sterility test

The sample for the final container and first repeat final container sterility tests should be not less than 20 final containers from each filling of each lot, selected to represent all stages of filling from the bulk container. If the amount of material in the final container is 1.0 ml or less, the entire contents should be tested. If the amount of material in the final container is more than 1.0 ml, the volume tested should be the largest single dose recommended or 1.0 ml, whichever is larger. In addition to fluid thioglycolate medium used as described under *Sterility test on bulk vaccine* (above), a test should be performed in fluid Sabouraud's medium for the detection of mould contaminants. Incubation for this medium should be 20°-25°C with an observation period of not less than 10 days; a period of up to 21 days is necessary for the development of some contaminants. Experience has indicated that the volume of inoculum and medium should be adjusted so that mercurial preservatives will be diluted to less than 1 part in 7 million in order to prevent inhibition of growth in this medium. As an alternative, fluid thioglycolate may be used and incubated at 20°-25°C.

Potency

See chapter 18, page 145.

Dating

The expiry date is not more than six months after the date of manufacture or date of issue. The date of manufacture is the date of the initiation of the last satisfactorily passed potency test. The date of issue may be three months after the date of manufacture, provided that the vaccine has been stored at 2°-5°C by the manufacturer for this period. The product should be stored at 2°-5°C at all times. Freezing of phenolized rabies vaccines destroys the potency.

ULTRAVIOLET-LIGHT-IRRADIATED VACCINE

KARL HABEL [1]

Principles and Methods

The use of ultraviolet (UV) energy to inactivate micro-organisms without impairing their antigenicity is a well-established procedure.

UV energy has little ability to penetrate biological substances since it is absorbed rapidly. Therefore, for effective exposure, the material being irradiated must be presented to the incident UV rays in a very thin film. The use of UV-irradiated rabies vaccine became a practical possibility only after the development of apparatus that would accomplish this thin-film type of exposure. It is also well known that as the amount of UV energy absorbed by biological material increases, the degree of chemical change in that material increases, so the length of exposure must be as short as possible while accomplishing the purpose of inactivation, otherwise breakdown of the antigen will occur. The biological effect of UV irradiation on viruses is, for all practical purposes, instantaneous and once this immediate effect has taken place, no further action ensues. There is no evidence that any secondary by-products are formed in the irradiation process which would subsequently be deleterious to the antigen.

Besides the necessity for a controlled length of exposure and for the material to be irradiated in a thin film, the practical production of large volumes of vaccine requires that the apparatus used should allow for a continuous flow of the antigen suspension through the equipment. Although quartz is a good conductor of UV energy and has been used extensively for making various types of container in which an antigen could be exposed, in general an apparatus designed to eliminate the passage of the UV rays through quartz gives more efficient radiation. Ease of operation and the use of a readily available, inexpensive source of UV are, of course, other desirable features of any practical irradiation apparatus.

Apparatus

Several types of apparatus have been designed which in some way incorporate the principles listed above. Two types are available commercially in the USA and both have been proved to be satisfactory for the

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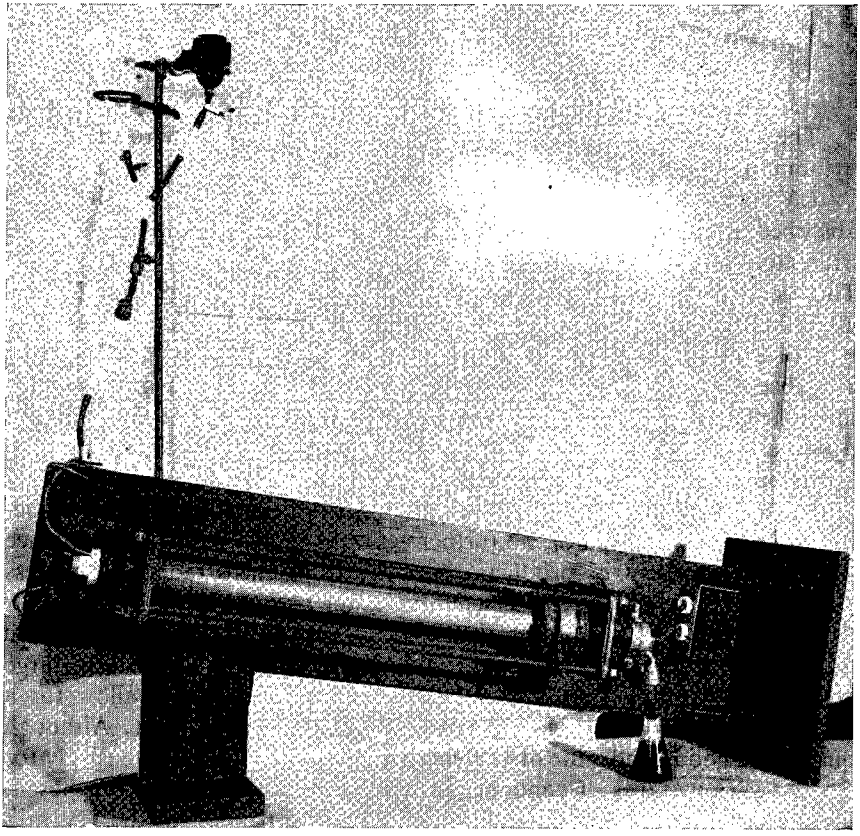
production of potent rabies vaccines. The "Dill" apparatus [1] employs a standard high-energy germicidal lamp that has a long effective life and is easily available and relatively inexpensive. The "Oppenheimer-Kettering" apparatus [2] uses a special, highly efficient lamp which is more expensive.

FIG. 1. CONTINUOUS-FLOW UV-EXPOSURE APPARATUS



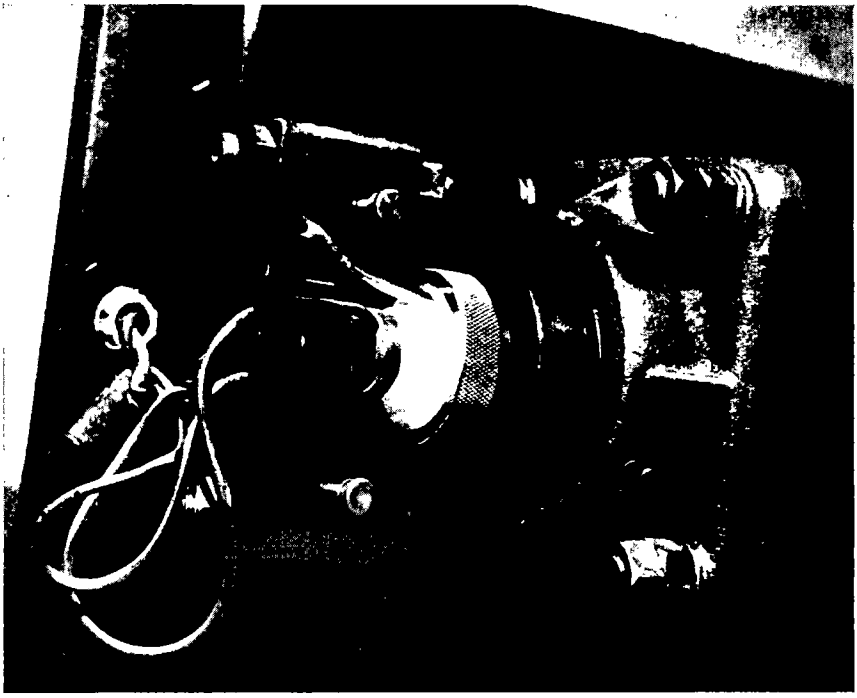
However, the construction of the type of apparatus of which the Dill machine is a modification can be accomplished by any modern machine-shop from basic materials that are readily available and not excessively expensive. The shop-made model used in our laboratory consists of a long hollow metal or glass tube, preferably of stainless-steel, so that the inner surface over which the virus suspension flows is very smooth and

[1] Dill Irradiator, J. J. Dill Co., 1302 Bixby Road, Kalamazoo, Mich., USA.

[2] Oppenheimer-Kettering UV Irradiator, Beckman Instruments, Inc., Fullerton, Calif., USA.

easily cleaned. This tube is of such dimensions that a germicidal lamp suspended through the centre of it will have one centimetre of clearance from the inner wall. This tube is fitted to slide smoothly into an outer, more sturdy, tube made of brass, which is mounted in bearings at each end. These tubes are to be rotated at a speed between 500 and 1000 rev/min, so a driving force must be applied. This may be accomplished by a gear- or

FIG. 2. INTAKE END OF UV-EXPOSURE APPARATUS



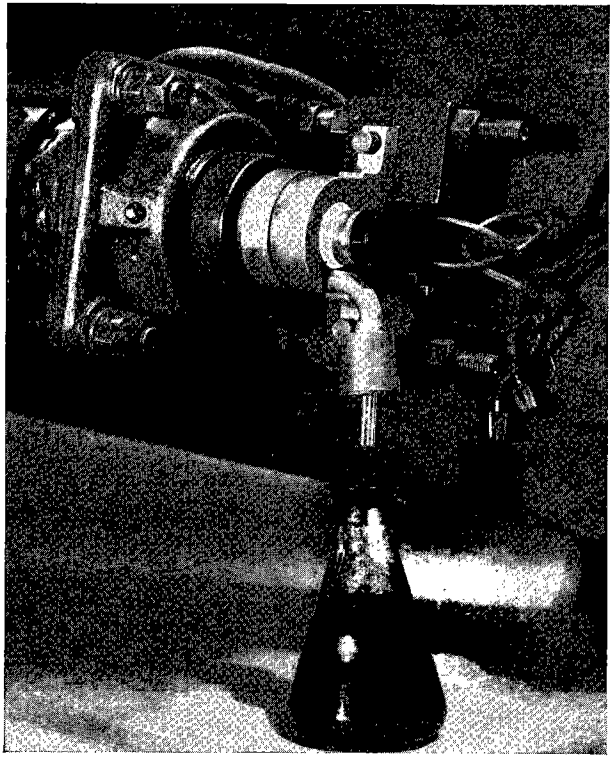
belt-drive, but in our own apparatus it is achieved rather simply by making the outer brass tube the armature of a small AC motor of the type used in electric fans. For long runs in large-scale production this may result in greater heating of the tube.

A suitably designed collecting-cup fits tightly to the discharge end of the rotating tube for collecting the vaccine as it flows through the apparatus. A supporting frame holds the rotating tube at an angle of 20° and also serves as the place of attachment for the sockets of the UV lamp. The virus suspension is introduced through a 16-gauge (1.65-mm) needle against the inner side of the rotating inside tube, where it is immediately spread in a film approximately 1 mm in thickness which progresses the length of the tube. The film is thus continuously exposed to UV light and is collected

through a closed connexion into a flask at the opposite end. A burette or inverted flask may be used as the original reservoir of virus suspension, and is connected to the intake-needle by rubber tubing with a valve for adjusting the rate of flow inserted in the line (see Fig. 1-3).

Some difficulty is occasionally experienced in maintaining a uniform flow rate when the flow by gravity is regulated by a valve in the line. This

FIG. 3. COLLECTING END OF UV-EXPOSURE APPARATUS



can be overcome by the use of a finger pump in which the solution is "milked" through the tubing at a constant rate.

The lamp used in this particular apparatus is of a germicidal type, 30-watt, and 36 in (91.5 cm) in length.

With the rates of flow necessary for the proper exposure in this type of apparatus, there is little opportunity for the heat from the UV lamp to be absorbed by the flowing emulsion. Actual thermocouple measurements show that the temperature of the emulsion increases by only 1°-2°C in the usual run. However, where large batches (over 500 ml) are irradiated in one run a check should be made on possible heating of the apparatus.

Standardization of Apparatus

From the practical standpoint the only standardization necessary is the determination of the minimum exposure (or maximum rate of flow) necessary to inactivate the standard rabies-virus emulsion for routine use in vaccine production. In the apparatus described, a single run with the following rates of flow should give a range of exposure from one completely inactivating the virus to one in which virus is still viable :

50 ml per minute
100 ml per minute
200 ml per minute
400 ml per minute.

If it is desired to determine actual exposure-time, this may be calculated after finding the volume of material in the rotating tube at any one time. A simple method of determining this volume is by the introduction of a dye into the intake end of the tube while test emulsion is being run. Collection of the sample should be started at the exact time the dye is introduced and stopped on its first appearance at the collecting end. The volume of this sample is the approximate amount in the rotating tube. When this volume, the total volume of any run, and the total time of that run, are known, the following formula will give the exposure-time :

$$\frac{\text{tube volume (ml)} \times \text{total time (minutes)}}{\text{total volume (ml)}} = \text{exposure-time (minutes)}.$$

Once the minimum exposure regularly causing virus inactivation has been established, the rate of flow should be set so that, for routine production, twice this amount of exposure will take place. This gives a satisfactory safety factor beyond the point of bare inactivation of the virus, and yet well within the limits of exposure possible before destruction of the antigen with loss of immunizing potency. It has been found that five times the minimum inactivating exposure is necessary before any marked drop in antigenic potency occurs.

Under ideal operating conditions there should be a continuous monitoring of the amount of UV energy to which the emulsion is being exposed. Such a device is available for attachment to the exposure apparatus.[1]

Virus Emulsion

All the factors of importance in rabies-vaccine production by any other method of inactivation are of equal importance for material to be irradiated.

[1] UV Irradiation Controller, Archer Reed Co., 23874 Kean Avenue, Dearborn, Mich., USA.

The virus titre of the original emulsion should be as high as possible, but variations of as much as one in the log titre will not noticeably change the necessary exposure-time. Since UV light will not penetrate biological materials to any appreciable depth, it is quite important that the emulsion be a uniformly fine one. To ensure this it is desirable to screen the emulsion through several layers of sterile surgical gauze or a wire screen.

In general, a 5% whole emulsion of infected nervous tissue is used with the irradiation equipment, but no difficulty is encountered in inactivating suspensions up to 10% in concentration. Virus suspension from sources other than brain emulsions can also be inactivated in this apparatus.

Preservation and storage of irradiated vaccine

If irradiated vaccine is to be kept in the liquid state before use, a chemical preservative should be added. Thiomersal (merthiolate) at a 1 : 8000 dilution, or 0.25% phenol, appears to be superior to formaldehyde. Irradiated vaccine, because it contains no substance in itself deleterious to viral antigen, may be kept frozen or may be dried from the frozen state. Freeze-drying will cause an immediate drop in immunizing potency, but the vaccine will then hold that potency indefinitely if sufficiently dry.

Testing of irradiated vaccine

Sterility, safety, and potency tests should be carried out on the irradiated vaccine in the same manner as with other types of inactivated rabies vaccines.

In general, it has been found that highly potent vaccines can be made more consistently by the irradiation method than by any other method of inactivation. There is no evidence to date that the incidence of post-vaccinal complications is any greater after the use of irradiated vaccine than with other types.

Steps in an irradiation run

(1) The inner tube is cleaned with soap or detergent solution, thoroughly rinsed, and then cleaned with ether.

(2) The apparatus is assembled, and the UV lamp inserted through the tube and clamped into its sockets.

(3) The collecting end-piece, which has been autoclaved, is connected to the apparatus and, through sterilized rubber tubing, to the collecting flask.

(4) Two sterilized reservoirs, one with sterile distilled water and one with virus emulsion, are connected by a Y-tube to the rubber tubing going to the intake-needle through a flow-valve or through a finger pump.

(5) The intake-needle is adjusted to direct flow against the inner side of the rotating tube, and is fixed in that position.

(6) The UV lamp is turned on for 5 minutes to sterilize the inside of the rotating tube, the motor of which has been started.

(7) Distilled water is run through the apparatus to wet down the tube, and the flow is adjusted to the desired rate.

(8) The flow of the distilled water is stopped, and that of the virus emulsion started. The rate of flow is rechecked. As soon as the emulsion appears at the collecting end, a fresh collecting flask is attached, and timing of the run started. The time is again taken at the end of the run, and the volume of collected emulsion is measured.

(9) When a series of exposures is being made to determine the maximum flow rate giving inactivation of the virus, the sequence should be from the longest to the shortest exposure.

CHICKEN-EMBRYO VACCINE

HILARY KOPROWSKI [1]

At present the Flury and the Kelev strains of rabies virus adapted to and modified in the developing chicken embryo are used for the production of chicken-embryo rabies vaccines employed in the prophylactic immunization of dogs.[2] Vaccine made with the Flury strain (Koprowski & Cox, 1948) has been used on a worldwide basis and therefore the description of the production procedure will be mainly confined to the Flury-strain vaccine. The same principles, however, are followed in the preparation of chicken-embryo rabies vaccine with the Kelev strain (Komarov & Horenstein, 1953).

The Flury-strain vaccine for dogs represents the 40th-50th egg-passage level of the virus. At this egg-passage level the Flury virus is fully virulent by the intracerebral route for mice, cotton-rats, hamsters, and guinea-pigs, while rabbits are more resistant. Hamsters, mice, and cotton-rats are susceptible to extraneural injection, but guinea-pigs are more resistant. On the other hand, rabbits and dogs fail to show signs of infection when inoculated intramuscularly with concentrated suspensions of the virus.

Further modification of the Flury strain has been achieved by continued egg passage (180th passage), and this has resulted in a lessened pathogenicity for experimental animals while its antigenicity has apparently been retained. For the present the high egg-passage level vaccine has been recommended for use in cattle and cats and the low egg-passage level vaccine, described here, for use in dogs only.

The Kelev chicken-embryo vaccine represents the 60th-70th egg-passage level of the virus. At this egg-passage level the virus does not produce any signs of infection in hamsters, guinea-pigs, or rabbits inoculated either extraneurally or intracerebrally. Suckling mice are, however, susceptible when inoculated intracerebrally. Dogs inoculated intramuscularly with concentrated suspensions of virus fail to show signs of infection.

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[1] Director, Wistar Institute, Philadelphia, Penn., USA. The author has taken over much of the material of the corresponding chapter of the first edition, which was contributed by the late Dr A. Komarov, Israel.

[2] These strains are available to national laboratories on request from the World Health Organization, Geneva, Switzerland.

Preparation of seed material

At the proper egg-passage level of the virus (Flury, 40th-50th, and Kelev, 60th-70th egg-passage level), a sufficient quantity of 60% embryo suspension in distilled water is prepared, tested for potency (see chapter 19, page 152) and preserved either frozen or in its dehydrated form. This constitutes the seed material. Just before use a 20% suspension of seed material in distilled water is prepared and tested for viability as follows.

Inoculate a group of six mice intracerebrally with 0.03 ml of a 10^{-1} dilution of the seed material, using a 0.25-ml tuberculin syringe with a $^1/_4$-in 27-gauge (0.40-mm × 6-mm) needle. If the seed material contains live virus the mice will sicken on the 6th-8th day. Should inoculated mice remain unaffected by the 9th day, discard the inoculated eggs.

In the case of the Kelev strain the viability test must be carried out in suckling mice.

Inoculation of eggs

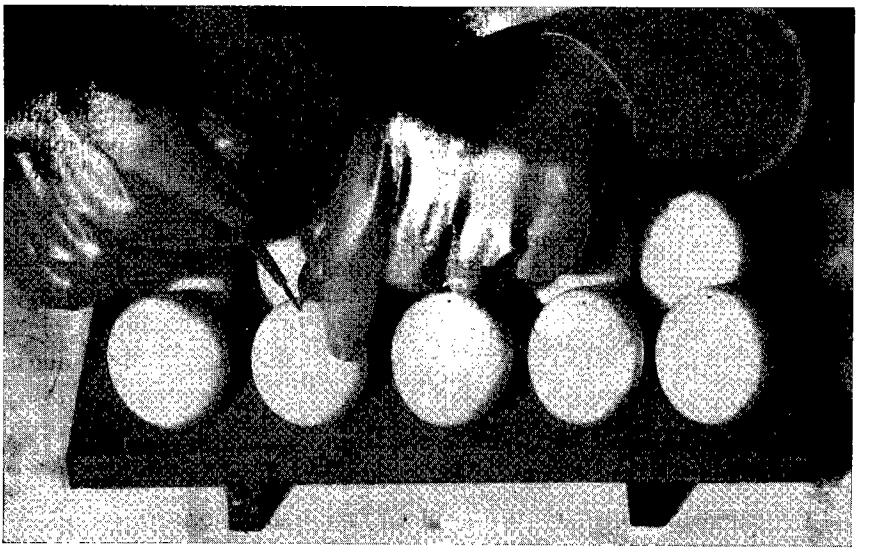
The number of eggs to be inoculated will depend on the amount of vaccine required and on the storage facilities. On a large production scale the average yield is 4-6 dog-doses per harvested embryo. Embryo mortality due to non-specific causes does not usually exceed 15%.

Fertile hen eggs incubated for 7 days are used. It is best to obtain fertile incubated eggs for vaccine production from a commercial hatchery and transfer the eggs to the laboratory one day before inoculation. On the 7th day of incubation the eggs are candled ; unfertilized eggs and those with dead or weak embryos (poor vascularization, sluggishness), and eggs showing displaced air sacs are discarded. While candling, the boundaries of the air sacs are marked on the shell with a pencil and the eggs are arranged air sac uppermost in a tray (a commercial cardboard egg-tray serves the purpose well). The shell is painted at the top of the air sac with 70% alcohol, and flamed briefly.

By means of a carborundum disc attached to a rotary motor tool or a dental drill, make two crosswise cuts, or penetrate the shell directly at the top and centre of the air sac. If this type of cutting machine is not available, pierce the shell at the point indicated by means of any sharp instrument (see Fig. 1).

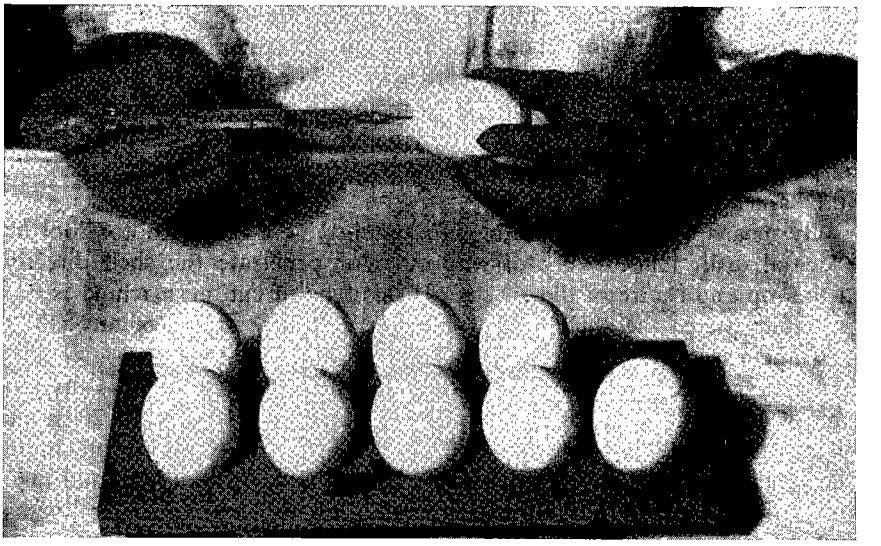
The eggs are inoculated in the yolk sac with 0.25 ml of the 20% suspension of seed material, using a syringe with a $^3/_4$-in to 1-in 19- to 20-gauge (6-25-mm × 0.90-1.10-mm) needle. Fill the syringe with the inoculum and hold it in the right hand parallel to the table ; with the left hand take an egg from the tray and hold it parallel to the table on the same line as the syringe, and with the blunt end of the egg directed toward the syringe. Through the cut in the shell introduce the entire length of the needle into

FIG 1. PIERCING THE AIR SAC



By courtesy of Dr F. Perez-Gallardo, Madrid

FIG. 2. YOLK-SAC INOCULATION OF EGG IN HORIZONTAL POSITION



By courtesy of Dr F. Perez-Gallardo, Madrid

the egg and deposit 0.25 ml of the inoculum (see Fig. 2). The inoculated eggs are replaced in the tray. On completion of the inoculation the seed material is tested for bacterial sterility, and the cut portion of the shell is sealed by means of a melted mixture of paraffin and vaseline (two-thirds paraffin, one-third vaseline), or collodion.

After inoculation the eggs are incubated at 36.5°C for a period of 9-10 days.

Harvest

At the end of the incubation period, only living embryos are harvested. Yolk, yolk-sac, extra-embryonic tissue, and fluid are discarded.

The eggs to be harvested are arranged in a tray, and the shells are sprayed or painted with 70% ethanol and flamed.

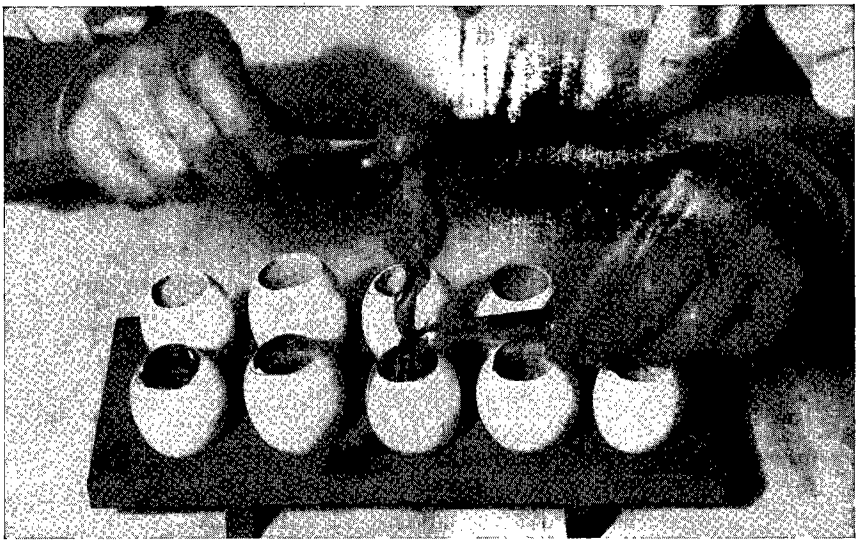
The live embryos are harvested by one of the following methods:

(*a*) Cut off the shell over the air sac by means of sterile sharp-pointed scissors (see Fig. 3). Tear away the membrane, thus exposing the embryo. Pass a wire hook attached to a handle under the neck of the embryo,

FIG. 3. CUTTING OFF SHELL COVERING AIR SAC

By courtesy of Dr F. Perez-Gallardo, Madrid

FIG. 4. SEPARATING EMBRYO FROM MEMBRANES



By courtesy of Dr F. Perez-Gallardo, Madrid

and pull the embryo slowly upward. The embryo will be pulled out of the egg, free from the yolk and extra-embryonic material.

Alternatively, lift the embryo out with one pair of forceps while separating the membranes with another (see Fig. 4).

(*b*) Put on sterile rubber gloves. Crack the shell of the eggs at the centre by means of a sharp sterile instrument. Open the shell with gloved hands and pour the contents over a sterile wire gauze. The fluid and yolk should drop through the meshes into a sterile container placed under the wire gauze layer. Collect the embryos from the layer of gauze into a proper container.

Preparation of embryonic suspension

The harvested embryos are placed in a chilled weighed container. The total weight is noted and then the embryos are transferred to a chilled Waring blendor. Sufficient chilled sterile distilled water [1] is added to the blendor to prepare a 66% embryo suspension (5 ml of distilled water for every 10 g of embryos). The embryos are ground three or four times, each time for about 3 minutes, and the blendor is chilled between each grinding.

[1] If it is found that contamination occurs, 1000 IU of penicillin and 2 mg of streptomycin per ml should be added to the water.

The resulting suspension is filtered by being squeezed through two or three layers of gauze into a chilled container. It is then measured and diluted with an equal volume of a stabilizer [1] to give a final embryo concentration of 33%. The vaccine is placed in ampoules or bottles so that each ampoule or bottle contains 3 ml of 33% suspension, which equals one dog dose.

Desiccated vaccine

The embryo emulsion is desiccated from the frozen state. After desiccation, the ampoules are sealed either in vacuum or in an atmosphere of nitrogen and stored at 4°C. The addition of a stabilizer to the vaccine before desiccation greatly increases the resistance of the virus to thermal inactivation.

Bacterial safety tests

Tests for the presence of pathogenic organisms are made in mice. Eight young adult mice are each inoculated intraperitoneally or subcutaneously, with 0.5 ml of the restored vaccine. At least 7 of these mice must remain alive and healthy for a 7-day observation period.

A sample of bulk material collected from each batch before the addition of antibiotics should be tested by classical bacteriological techniques for the presence of *Salmonella*.

Specific safety test

A specific safety test on the product is made in at least 2 young dogs. Each dog receives intramuscularly the recommended field dose and is observed for 21 days. For a test to be considered satisfactory, all dogs must remain free of any symptoms that might be attributed to the vaccine. The dogs used in this test should not be used in any subsequent safety test.

Virus titration in mice

1. Each of 2 vials of vaccine representing each series or subseries in final containers (irrespective of the manufacturers' recommended dose)

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[1] Preparation of a stabilizer for desiccation of the Flury vaccine (formula adapted from the one used by the Onderstepoort Laboratory, Republic of South Africa):

In one litre of distilled water dissolve:

KH_2PO_4	2.7 g
Peptone (Difco-Bacto)	20.0 g
Lactose	100.0 g

This mixture must be heated slightly in order to dissolve the lactose. Cool the mixture to room temperature and adjust the pH to 7.6 with a normal solution of NaOH (the amount needed depends on the brand of peptone used; 22 ml are needed for the above brand). Sterilization is obtained by filtration through Seitz or millipore filters under positive pressure; do not autoclave.

is reconstituted with sterile distilled water containing 2% normal horse serum (3 ml of the diluent per dog dose of dried vaccine). Withdraw a half dog dose (1.5 ml) from each of the 2 vials of restored vaccine, or a full dog dose (3 ml) if the 2 vials of restored vaccine are combined, and add this to 7 ml of distilled water containing 2% normal horse serum in a test tube. This gives 10 ml of diluted vaccine, representing a 10^{-1} dilution.

Antibiotics may be added to the diluent at the discretion of the manufacturer. Penicillin 500 IU and streptomycin 1000 μg (1 mg) shall be added per ml of diluent for each dilution.

2. Using prescribed and acceptable laboratory procedures, make further 10-fold dilutions from the 10^{-1} dilution prepared above. A suggested method is as follows:

(*a*) Prepare an appropriate number of test tubes with 4.5 ml of the sterile diluent referred to above.

(*b*) Using a 1-ml pipette, transfer exactly 0.5 ml of the reconstituted well-mixed sample (10^{-1} dilution) to the first tube without touching the diluent with the pipette. Discard the pipette in an appropriate receptacle.

(*c*) Take another 1-ml pipette and mix the suspension in the first tube by drawing and expelling the suspension in and out of the pipette at least 5 and, preferably, 10 times. The mixing should be done as vigorously as possible without causing bubbles to form extensively. Using the same pipette, withdraw enough of the mixture in tube 1 to transfer 0.5 ml to tube 2 without touching the diluent with the pipette. Discard the pipette. Continue this procedure until all the tubes have been used.

(*d*) The reconstituted vaccine (10^{-1} dilution) is mixed with the sterile diluent as prescribed above to give the following dilutions:

Tube No.	*Volume of diluent (ml per dog dose of dried vaccine)*	*Volume transferred (ml)*	*Resultant dilution*
	10		10^{-1}
1	4.5	0.5	10^{-2}
2	4.5	0.5	10^{-3}
3	4.5	0.5	10^{-4}
4	4.5	0.5	10^{-5}
5	4.5	0.5	10^{-6}

Tubes containing the desired dilutions are placed in an ice-bath. The highest dilution to be used is optional; however, the lowest dilution used should kill at least 80% of the mice. At least 10 young adult mice (4 to 6 weeks of age, approximately 16 grams) are inoculated intracerebrally with 0.03 ml of each dilution, progressing from the highest to the lowest dilution. At least 80% of the mice inoculated for each dilution must survive longer than 4 days or the test is considered invalid and must be repeated.

(Test mice that succumb during inauguration of the test may be replaced and disregarded in determining the percentage that survive the 4-day period.)

Deaths occurring during the first 4 days are not considered in the test. The mice are observed for 14 days, death and paralysis subsequent to the first 4 days are recorded, and the LD_{50} titre calculated by the Reed & Muench method (see chapter 16, page 142). A satisfactory vaccine shall have a titre of not less than $10^{3.3}$ LD_{50} per 0.03 ml. This minimum level is to be maintained throughout the expiration period. A suggested minimum titre for release is 0.5 log higher, i.e., $10^{3.8}$ LD_{50} per 0.03 ml.

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THE PREPARATION AND LYOPHILIZATION OF ANTIRABIES VACCINE IN SUCKLING RATS

M. K. KARAKUJUMČAN,[1] *B. M. PARIZ*[2] *& V. D. SOLOV'EV*[3]

An important property of vaccine preparations is that they should be able to maintain their specific activity over a lengthy period, not only at optimum temperature but also under less favourable environmental conditions. This is particularly true of antirabies vaccine, since it may have to be transported at any time to any place where it is urgently needed.

Liquid antirabies vaccines remain fit for use for a comparatively short period only. They are issued with a statement concerning the expiry date (see chapter 11, page 116) and the storage temperature, which must not exceed 5°C. This makes their storage, transport and use appreciably more difficult. Freezing the vaccine or heating it above 37°C causes speedy coagulation of the tissue substrates and make the preparation unfit for vaccination purposes.

The method of freeze-drying has long been in use in the production of bacterial and viral vaccines, but until recently antirabies vaccines have been produced only in liquid form. Nevertheless, a number of investigations have been carried out that demonstrate the possibility of successfully drying vaccines prepared both from the live fixed virus and from the inactivated virus. An analysis of the results of the work done in this direction in the USSR leads to the conclusion that quite satisfactory results can be obtained from lyophilization of antirabies vaccine made from brain tissue if a solution containing 15% of sucrose and 1% of gelatine is added as a stabilizer. Apparently, dried antirabies vaccine has not found practical application hitherto because of the fear of intensifying the complications that sometimes arise following antirabies vaccination. We therefore thought that it would be useful to devise a method of producing dried antirabies vaccine by using what is known as the "allergen-free" antirabies vaccine prepared from the brain tissue of new-born rats. The research done by

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Kabat et al. (1946, 1947) and by Svet-Moldavskij and his associates (1959, 1960, 1962, 1965), served as a basis for this technique. Independently of these studies in the USSR, the production of an allergen-free antirabies vaccine from the brains of suckling mice has been described by Chilean workers (Fuenzalida & Palacios, 1955 ; Fuenzalida et al., 1964).

Suckling rats aged four to seven days are used for preparing the lyophilized antirabies vaccine. The Pasteur strain of fixed virus, which has undergone 3249 passages through rabbit brain, is used for infection. A sterile suspension of the brain of an infected rabbit is administered to the rats intracerebrally in a dose of 0.03 ml and a dilution of 1 : 100. After infection, the rats are put back in the cages with the nursing females. Seventy to seventy-four hours after infection animals showing symptoms of rabies are killed with ether vapour. The skull is opened under sterile conditions and the brain is removed and placed in a test-tube with 3 ml of a 1% solution of phenol in distilled water. The cerebellum is inoculated on sugar broth to test for sterility.

Until the sterility test has been completed, the brain is kept at a temperature of 4°C. The sterile brain tissue is then homogenized and a 1% solution of phenol in distilled water is added to the homogenate until a 20% brain suspension is obtained. This suspension is placed in the incubator for 14 days at a constant temperature of 22°C in order to inactivate it.

The initial concentration of virus in the brain suspension is quite high (corresponding to a 50% end-point dilution of 10^{-6}—10^{-7}), which demonstrates that it multiplies intensively in the brain of new-born rats. The reduction in infectivity of the brain suspension proceeds quite quickly during the first three days of inactivation and then slowly up to the fourteenth day.

After inactivation an equal volume of a suspending medium containing 15% of sucrose and 1% of gelatine dissolved in distilled water is added to the 20% brain suspension. Thus the vaccine consists of 10% brain suspension, 7.5% sucrose and 0.5% gelatine. This vaccine is dispensed in 1.5-ml quantities into ampoules and is then frozen at a temperature of —45° to —50°C and quickly loaded into the chamber of a vacuum-drying apparatus.

Lyophilization of the vaccine is carried out for 26 to 29 hours at a residual pressure decreasing from 0.01 to 0.003 Torr. The apparatus used for heating the lyophilized material up to 26°-28°C is switched on between the sixth and eighteenth hour following the beginning of lyophilization and this temperature is maintained until the end of the process.

After lyophilization the ampoules are flame sealed, care being taken to preserve the vacuum. The residual moisture content of the lyophilized vaccine is determined by heating weighed portions of 100-150 mg at 100°C for 1 hour in vessels of 7-8 ml capacity. The mean of two or three determinations should be less than 2%.

An innocuity test should be performed on the lyophilized vaccine in the same manner as described for Semple-type vaccine in chapter 11 (page 115). Lyophilized vaccine does not cause the development of allergic encephalitis in guinea pigs.

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PART III

VACCINE POTENCY TESTS

CHAPTER 15

GENERAL CONSIDERATIONS IN POTENCY TESTING

KARL HABEL [1]

Although the need for evaluating the immunizing potencies of rabies vaccines has been recognized since the early Pasteur days, and practical standardized tests have been available and in use for over 20 years, many laboratories producing vaccines still do not make a routine practice of potency testing their products. It has long since been established that merely following a described standard vaccine production procedure does not necessarily assure vaccines with consistently satisfactory potency levels. Many laboratories that had been producing vaccines for years have been amazed on first testing to find their products with low or negligible potencies.

It has always been difficult to evaluate vaccines on the basis of results obtained in human beings under field conditions because of the lack of true controls, the relatively small number of human cases of rabies in a given area, and the impossibility of taking into account all the many variable factors that tend to make human exposures not comparable. Furthermore, the need is really for an evaluation of a vaccine *before* it is released for human or veterinary use.

There would appear to be three important considerations in assessing any potency test of rabies vaccines. First, the test procedure should actually evaluate the property of the vaccine that determines its effectiveness in the prophylaxis of rabies in man or animals. Using a naturally susceptible host, the ideal test would simulate conditions of natural exposure and usual prophylactic treatment. This would mean the use of street virus introduced through a bite-wound, followed by daily doses of vaccine in the case of those vaccines intended for human use. This has, of course, been found to be impracticable, as have most types of test where administration of vaccine is started *after* experimental exposure of the test animal. Most tests, therefore, involve multiple doses of vaccine (as administered in man) followed by subsequent challenge with fixed virus given intracerebrally, as being a more easily standardized type of challenge. While far from reproducing the situation with natural exposure and the standard schedule of vaccine administration, this type of test has been shown to

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reflect fairly closely the ability of a vaccine to protect under natural conditions. On the other hand, the chicken-embryo live virus vaccines, as used in veterinary practice, are administered prior to exposure in a single dose and depend upon multiplication of the attenuated virus for their immunizing effect. Therefore, potency tests of these vaccines require the use of animals in which the vaccine virus strains are likewise avirulent but capable of multiplying. The test involves a single dose of vaccine followed by a subsequent challenge with virus introduced by the peripheral route.

The second important factor in a potency test is its practicability—the ease with which it is carried out, the availability of the material used, and the cost and time involved. Not all laboratories can obtain large numbers of experimental animals easily, nor can they, perhaps, support the expense of repeated tests when animal costs are high. The time factor is important, since newly prepared vaccine must be held until potency tests are complete and the time required for this has to be deducted from its period of effectiveness.

The third requirement is for standardization of the test procedure so that there will be comparability of results from one vaccine to another in a single laboratory and between different laboratories.

Through experience it has been found that the two most important variables that can markedly influence the results of any type of rabies potency test are the animals and the challenge virus employed. Both good general health and the proper age of the animals are necessary for optimal results. Uniformity of response among the individual animals making up the test group does not necessarily require highly inbred strains but all animals should be from the same closed colony stock. Another animal strain factor that has proved troublesome in the mouse tests is the variation from colony to colony in the ability of the mice to respond with a high degree of immunity to a given vaccine. Obviously, for test purposes a strain of mice should be used that has been shown to respond efficiently to a known potent vaccine.

Variation in the degree of demonstrable immunity has also been shown to depend upon the strain of virus used to challenge the immunized animals. This variable has been eliminated by the development of a standard challenge virus—the CVS fixed virus derived from the original Pasteur strain.[1] The proper technique for preparing and handling the standard challenge virus is given in chapter 18 (page 145).

In the following sections of Part III five potency tests are described. The first three are mouse tests primarily used for testing inactivated virus vaccines intended for human use. They are also satisfactory for potency tests of inactivated vaccines for veterinary use. The decision as to which

[1] CVS is available to national laboratories on request from the World Health Organization, Geneva, Switzerland.

of these three tests to use in any particular laboratory will depend on the type of information required and the facilities available. If a laboratory has limited facilities for animal procurement, and wishes merely to screen its vaccines for potency or lack of potency, then the non-quantitative modified Habel test should be used. If facilities are available and a quantitative evaluation of potency is desired as a continuing check on efficiency of vaccine production, then one of the more standardized tests is indicated. The original Habel test (chapter 15) and the NIH test (chapter 17) are both quantitative; the former is of the immunity-breakdown type where all animals receive the same immunization and the challenge virus dose is varied, whereas the NIH test is of the antigen-extinction type where varying amounts of vaccine are used and all mice receive the same challenge. Both these quantitative tests require larger numbers of mice and should include a standard vaccine run in parallel with the unknown under test. The inclusion of a standard vaccine control is absolutely essential in the NIH test. Under ideal conditions, the antigen-extinction NIH test is the most standardized and gives the statistically most significant results.

The two tests described for use in testing modified live-virus vaccines (chapters 19 & 20) also represent a screening, non-quantitative test in guinea pigs and an antigen-extinction quantitative test in mice (NYLAR test). Like the NIH test, the NYLAR test requires the use of a standard control vaccine.

Should a laboratory wish to test its vaccine in the species in which it will eventually be used in practice, this can be done directly with veterinary vaccines. Immunization of the species can be done as in the field, followed by challenge intramuscularly with street virus including an adequate number of vaccinated and control animals to give statistically significant results. However, this is a difficult procedure which should be carried out only by a laboratory actively engaged in rabies research. No such direct test, of course, can be done in man, so the nearest approach is to immunize humans according to the recommended schedule and bleed the individuals at intervals to determine the rapidity and degree of neutralizing antibody response.

HABEL TEST FOR POTENCY

KARL HABEL [1]

Mice

White Swiss mice, 4-6 weeks of age, and uniform in weight, are used; they may be selected at random, irrespective of sex, or mice of only one sex may be used if preferred.

Immunization Procedure

Sixty mice receive 0.25 ml of a vaccine diluted to give a 0.5% emulsion of original wet-weight of brain. Inoculations are given intra-peritoneally on Monday, Wednesday, and Friday during two successive weeks (a total of six doses). Thirty mice should be kept apart from the rest at the beginning of the immunization for use as controls at the time of challenge. When testing vaccines prepared from material other than brain tissue, the vaccine should be diluted 1 : 10 before inoculation of 0.25 ml per dose.

Challenge

A challenge test is performed on the 14th day from the first dose of vaccine. At this time two ampoules of the standard challenge fixed virus (see chapter 18, page 145) should be thawed out and diluted to a 10^{-1} suspension. Serial tenfold dilutions from 10^{-1} to 10^{-7} are then made, using 2% heat-inactivated horse or rabbit serum in distilled water as diluent. It is recommended that the dilutions of challenge virus be held in an ice-water bath during the performance of the test to prevent loss of virus titre. It is important to use a separate pipette for transferring and mixing the material at each step in the serial dilution series.

Groups of 10 vaccinated mice are then challenged intracerebrally with 0.03 ml of the 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} dilutions of virus in that order. A single syringe and needle may be used for all inoculations, provided the empty syringe is rinsed several times in the next dilution before being refilled.

[1] Chief, Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md., USA.

With a new syringe the control mice are then inoculated with the 10^{-7}, 10^{-6}, and 10^{-5} dilutions of virus in order to determine what dilution represents the LD_{50}. If the challenge virus is fully active, these three dilutions will usually give survival rates ranging from 100% to less than 50%. Scattered deaths at all three dilutions should be viewed with suspicion as indicating errors in the dilution of virus. For the potency test to be valid, the 50% end-point (dilution corresponding to 50% rabies mortality) should be beyond the 10^{-5} dilution in the control mice.

All mice are observed for 14 days, and only deaths occurring after the fifth day should be considered as rabies deaths. Mice should be observed daily and symptoms recorded. Mice surviving at the end of 14 days but showing definite symptoms of rabies should be considered as having died of rabies in potency calculations.

Determination of Degree of Protection

50% end-point dilutions are determined by the method of Reed & Muench,[1] as illustrated in the Annex. This gives the dilution in vaccinated and in control mice where, theoretically, 50% of the mice would die of rabies, based on the actual results in the test with the dilutions employed. By subtracting the log of the 50% end-point in the vaccinated animals from that in the controls, the log of the "LD_{50} of protection" is readily obtained. To meet minimum standards, the difference should be 3, or log 1000, indicating that the vaccine affords protection against 1000 LD_{50}.

Modification of Technique for Live-Virus or Attenuated Vaccines

Where the vaccination schedule prescribes gradually increasing doses, as in the original Pasteur type of vaccine, the 6 intraperitoneal doses of 0.25 ml of equivalent 0.5% emulsion should be made starting with the least virulent of the vaccine doses and grading to the most virulent. If all doses in the vaccination schedule are the same, then no change from the original procedure is necessary.

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[1] Reed, L. J. & Muench, H. (1938) *Amer. J. Hyg.*, **27**, 493.

Annex

PROCEDURE FOR DETERMINING 50% END-POINTS OF HABEL-TYPE POTENCY TEST IN MICE

Suppose that a typical protocol gives the following results :

	Vaccinated mice		*Cumulative totals*		
Dilution	*Survived*	*Died*	*Survived*	*Died*	*Percentage mortality*
10^{-1}	4	6	4	20	89
10^{-2}	5	5	9	14	61
10^{-3}	3	7	12	9	43
10^{-4}	8	2	20	2	9
10^{-5}	10	0	30	0	0

Totals are accumulated from 10^{-1} to 10^{-5} for survivors and from 10^{-5} to 10^{-1} for mice concidered as having died of rabies.

To calculate the 50% end-point proceed as follows :

$$\frac{50\% - (\text{mortality next below } 50\%)}{(\text{mortality next above } 50\%) - (\text{mortality next below } 50\%)} = \frac{50 - 43}{61 - 43} = 0.39$$

Now subtract this figure (0.39) from the log [1] of the dilution that gave the mortality next below 50% :

$$3.00 - 0.39 = 2.61$$

This figure (2.61) is the log of the 50% end-point for this protocol.

Next calculate the 50% end-point for the control mice in the same way :

	Vaccinated mice		*Cumulative totals*		
Dilution	*Survived*	*Died*	*Survived*	*Died*	*Percentage mortality*
10^{-5}	0	10	0	17	100
10^{-6}	4	6	4	7	64
10^{-7}	9	1	13	1	7

$$\frac{50 - 7}{64 - 7} = \frac{43}{57} = 0.75$$

$$7.00 - 0.75 = 6.25$$

[1] In making these calculations, the minus signs preceding the logarithms are disregarded.

The log of the 50% end-point for the controls is thus 6.25.

Now subtract the log of the 50% end-point in the vaccinated mice from the log of the 50% end-point in the controls :

$$6.25 - 2.61 = 3.64$$

The difference (3.64) is the log of the "LD_{50} of protection", i.e., this vaccine protected against $10^{3.6}$ LD_{50} or 4366 LD_{50}. The figure should be rounded out to the nearest hundred, i.e., 4400 LD_{50}.

MODIFIED HABEL TEST FOR POTENCY

KARL HABEL [1]

For many laboratories producing antirabies vaccine, the lack of relatively large numbers of experimental animals makes the routine use of the standard mouse test for potency impractical. Especially is this true in those laboratories where multiple small volumes of vaccine are prepared at frequent intervals. In these situations it would be of value to have available a simplified potency test which would screen out vaccines of below-standard antigenic potencies and which at the same time would be comparable with the more complete standard test in respect of minimum requirements.

In checking the results of many potency tests with vaccines of high, low, and intermediate levels of antigenicity, it has been apparent that approximately 500 LD_{50} as an intracerebral challenge dose kills less than half the immunized mice if the potency of the vaccine determined by the complete test is over 1000 LD_{50} of protection, which is the minimum requirement.

The technique of the test is the same as the standard procedure (see chapter 16, page 140) using six intraperitoneal doses of a 0.5% suspension of nervous tissue (0.25 ml), and intracerebral challenge on the 14th day followed by 14 days of observation. Only 20 mice are immunized, and 15 additional mice are held as controls. On the 14th day the standard potency-test challenge virus, diluted to contain 500 LD_{50} per 0.03 ml, based on past experience with a frozen virus pool, is given intracerebrally to the vaccinated mice, and at the same time groups of 5 control mice receive the challenge virus diluted to 10^{-5}, 10^{-6}, and 10^{-7} (see chapter 16, page 140, for an analogous procedure).

To be a valid test, the control titre should indicate that the challenge dose given to the vaccinated mice was between 100 and 1000 LD_{50}, and 50% of the vaccinated mice should survive in order that the vaccine pass the screening potency test (for calculation, see chapter 16, page 142).

It is recommended that at periodic intervals a complete standard potency test (see chapters 16 and 18, pages 140 and 145) be performed to obtain a more quantitative evaluation of the vaccine over long periods of production in each laboratory.

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POTENCY-TEST REQUIREMENTS OF THE UNITED STATES NATIONAL INSTITUTES OF HEALTH (NIH)

EDWARD B. SELIGMANN, jr, Ph. D.[1]

The Standard Challenge Virus

The standard challenge virus (CVS) is supplied periodically, usually once a year, to laboratories in the USA[2]. This provides a nearly uniform challenge virus and makes possible the evaluation of different lots of vaccines as well as of vaccines from different laboratories. Laboratories are urged to follow closely the procedures outlined.

The Working CVS

In the USA, CVS is supplied as a 20% mouse-brain suspension in a diluent containing 2% of horse serum in distilled water. This has been stored under dry-ice before shipment ; it should be used only if received in the frozen state. It is also distributed as a freeze-dried preparation when there is the possibility that the shipment will thaw before receipt. The ampoules of the dried preparation contain 0.5 ml of a 20% mouse-brain suspension otherwise identical with the frozen preparation. The contents of the ampoule of frozen virus should be thawed rapidly under cold running water and then diluted 1 : 2 with 2% horse-serum diluent. This yields a 10% suspension, which is centrifuged for 5 minutes at a relative centrifugal force of between 1000 and 2000 *g*. The supernatant fluid is diluted to yield a 10^{-3} dilution of the virus and, using 0.03 ml of this dilution as the inoculating dose, a sufficient number of normal mice are inoculated intracerebrally to produce the amount of working CVS needed for approximately one year. (One mouse brain will yield approximately 1.5 ml of a 20% suspension.) In order to ensure a high titre in the working preparation when starting with freeze-dried seed virus, it is best to make two mouse passages and use

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[1] Chief, Reference Standards Section, Division of Biologics Standards, National Institutes of Health, Bethesda, Md., USA.

Much of the text of this section has been adapted from United States National Institutes of Health (1953) *Minimum Requirements : Rabies Vaccine*, 3rd rev., Bethesda, Md., USA.

[2] CVS is available to national laboratories upon request from the World Health Organization, Geneva, Switzerland.

the second passage for the working CVS. Mice will show paralysis in 6 to 10 days. When an inoculated mouse is completely paralysed but still breathing the brain is harvested and immediately frozen with dry-ice. The harvested brains are placed in a common container and when the collection is complete they are thawed, weighed, and ground to pulp ; enough of the 2% horse serum diluent is then added slowly, while grinding, to yield a 20% suspension. The suspension is given a lot number and without straining or centrifuging it is distributed into ampoules. The ampoules are flame-sealed, quick-frozen and stored at dry-ice temperature (approximately −70°C).

Each step in preparing the working CVS must be carried out promptly so as to ensure the survival of the maximum possible amount of virus. Before use as challenge virus, the LD_{50} value of the lot should be determined in six-week-old mice. The lot is satisfactory provided that the LD_{50} value is between the $10^{-6.0}$ and $10^{-8.0}$ dilutions, inclusive. The maximum variation in the titre obtained from test to test should not exceed one tenfold dilution when the same lot of challenge virus is used. Storage may be as long as full potency is maintained as shown by mouse titration. This is essential in order to ensure uniformity of the working CVS among all laboratories producing rabies vaccine.

Reference Vaccine

A Reference Rabies Vaccine (ultraviolet-light-inactivated and freeze-dried) is distributed by the NIH to vaccine-producing laboratories in the USA. When the contents of each ampoule are resuspended in 8 ml of distilled water, the vaccine is considered to be a 10% suspension. The dried vaccine is stored at 2°-10°C at all times and after reconstituting to the liquid state should be used immediately.

Immunization of Mice

Three or more dilutions of each vaccine under test are prepared in buffered saline solution (0.85% sodium chloride in phosphate-buffer [1] solution pH 7.6), using five-fold increments. The range of dilutions is selected so that the middle dilution will contain enough vaccine to protect 50% of the mice. The range will depend upon the strength of the challenge dose, the kind of mice used, and the potency of the vaccine. With a challenge dose in the range 5-50 LD_{50} (see below) a dilution range of 1.0%, 0.2%, and 0.04% of brain tissue has worked well for the reference vaccine and commercial vaccines produced in the USA.

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[1] 15 ml of M/15 KH_2PO_4, 85 ml of M/15 $Na_2HPO_4.12H_2O$ and 900 ml of distilled water.

At least 16 mice approximately four weeks old weighing 11-15 g are injected intraperitoneally with 0.5 ml of each dilution of vaccine. Two doses of vaccine are given to each mouse one week apart. A new ampoule of the reference vaccine should be used for each dose. Enough control mice are set aside at the time the test mice receive the first dose of vaccine for an adequate titration of the challenge virus to be made with at least 10 mice for each dilution of virus (a total of 30-40 control mice).

Mice are vaccinated with 0.5 ml intraperitoneally (see accompanying figure). The needle best suited for vaccination is the 25-gauge ½- or $^3/_4$-inch (0.50-mm × 12- or 18-mm) needle. The vaccine suspension should be injected rapidly. If there is any doubt as to whether the needle point is in the peritoneal cavity, the point should be moved from side to side and the skin observed.

If a single needle and syringe are used for inoculating the mice with the vaccine, mice receiving the most dilute vaccine should be inoculated first followed by those receiving successively more concentrated vaccines. Unless each mouse is clearly marked, mice receiving each vaccine concentration should be housed separately from mice receiving other concentrations.

INTRAPERITONEAL INOCULATION OF A MOUSE

Challenge of Control and Test Mice

All mice are challenged intracerebrally 14 days after the first dose of vaccine.

One ampoule of the pooled working CVS is thawed rapidly under cold running water and diluted 1 : 6 with 2% horse serum in distilled water. This mixture is then centrifuged for 5 minutes at a relative centrifugal force of approximately 1500 *g*. The supernatant fluid is used to make further dilutions. All dilutions are made in the same diluent as originally used. It is recommended that the dilutions of the challenge virus be held in an ice-water bath, or its equivalent, during the performance of the test in order to prevent potency loss.

The immunized mice are challenged with a dilution of virus which has been found by routine tests to contain between 5 and 50 LD_{50} of virus. Each laboratory, therefore, must determine for itself the dilution of working CVS that will contain a test dose of between 5 and 50 LD_{50} of virus. The initial 1 : 6 dilution may be varied in accordance with experience. Serial tenfold dilutions of the supernatant are then made and the one found to contain the test dose of virus in 0.03 ml used for challenge. Experience in one laboratory with the working CVS prepared in that laboratory has led to the use of a 1 : 6 initial dilution and use of the 10^{-4} dilution of the supernatant for challenge.

After all immunized mice have been inoculated with the test dose of virus, one group of control mice is inoculated with the same test dose of virus. Three tenfold dilutions (10^{-1}, 10^{-2} and 10^{-3}), of the test dose are then prepared for injection of the remaining control mice. It is desirable to inject first the 10^{-3} dilution, then the 10^{-2} and lastly the 10^{-1} dilution of the test dose of virus. In this way one syringe may be used to challenge all the immunized mice and one group of control mice ; another syringe must be used to challenge the remaining control mice.

All mice are observed for 14 days from the time of the challenge injection. Only those deaths occurring after the fifth day and those preceded by signs of fixed-virus rabies (paralysis, convulsions) are considered deaths from rabies. However, any mice becoming paralysed but surviving the 14-day observation period are also counted as deaths from rabies.

The definitions of "paralysis" and "convulsions", as applied to mice following the injection of the challenge virus, are as follows :

Paralysis is the partial or complete loss of motor power of one or more legs.

Convulsions are indicated by violent and abnormal muscular contractions of the body—often termed spasms. These are brought about by external stimulation, such as a disturbing sound or handling.

For the test to be considered valid, the results obtained after challenge of the immunized mice must show that the dilutions of the reference vac-

cine encompass the 50% end-point—that is, the majority of the mice receiving the highest dose of vaccine survive, and the majority of the mice receiving the smallest dose of vaccine die. For the test vaccine, unless an exact end-point is especially desired, it is only necessary that the majority of the mice receiving the highest dose of vaccine survive. The virus titre of the test dose of challenge virus is not particularly important, provided that it falls between 5 and 50 LD_{50}. It is necessary, however, that all the control mice receiving the test dose of virus die. Because of the nature of the virus and the test involved, it is difficult to obtain exactly the same virus titre from one test to another.

Potency Test on Live or Attenuated Virus Vaccine

Vaccines containing live or attenuated virus, except for chicken-embryo vaccines (see chapter 19, page 152), can be tested in the same manner as killed-virus vaccines.

Calculation of Potency (Antigenic Value)

50% end-points are determined for both the reference vaccine and the test vaccine by the method of Reed & Muench. The end-point is calculated as a 50% effective dose (ED_{50}) to vaccine in milligrams of original brain tissue which will protect 50% of the mice. An abstract of this method is given in the Annex below. By dividing the ED_{50} of the reference vaccine by the ED_{50} of the test vaccine, a value is obtained which is the antigenic value of the test vaccine in terms of the reference vaccine. The LD_{50} of challenge virus received by the immunized mice is calculated by dividing the dilution of virus used as the dose by the 50 % end-point dilution of virus in control mice, as calculated by the method of Reed & Muench.

Antigenic value requirement (see below for computation)

The antigenic value of a vaccine under test should be 0.30 or higher in relation to the reference vaccine.

Annex

DETERMINATION OF 50% END-POINTS BY THE METHOD OF REED & MUENCH,[1] AS APPLIED TO THE POTENCY TEST OF RABIES VACCINE IN MICE

The 50% effective dose (ED_{50}) is calculated as the amount of brain tissue that will protect 50% of the mice against a subsequent challenge with rabies virus. The results of the challenge are arranged in columns

[1] Reed, L. J. & Muench, H. (1938) *Amer. J. Hyg.*, **27**, 493.

indicating the number of mice that survived and the number that died on each dose of vaccine. The cumulative totals for survived and dead are then calculated, proceeding from the high to the low dilutions for survivals and in the opposite direction for deaths.

EXAMPLE

Dilutions of 5% brain tissue vaccine	*Brain tissue (mg)*	*Number of mice*	*Survived*	*Died*	*Cumulative totals Survived*	*Died*	*Percentage mortality*
1 : 5	10	16	10	6	↑ 19	6	6/25=24
1 : 25	2	16	8	8	9	14	14/23=61
1 : 125	0.4	16	1	15	1	↓ 29	

From this tabulation, the 50% end-point dilution lies between 2 mg and 10 mg of brain tissue and is obtained by the following calculation : [1]

$$\frac{\text{(mortality next over 50\%)} - 50}{\text{(mortality next over 50\%)} - \text{(mortality next under 50\%)}} \times \text{log of dilution factor}$$

$$= \frac{61 - 50}{61 - 24} \times \log 5 = \frac{11}{37} \times 0.699 = 0.208$$

= logarithm of dilution at 50% end-point.

Antilog of dilution factor at 50% end-point multiplied by amount of brain tissue injected into mice at dilution at which more than 50% of mice died

= antilog 0.208×2.0 mg

= 1.61×2.0 mg = 3.22 mg = ED_{50}.

The antigenic value of a test vaccine is obtained by dividing the ED_{50} of the test vaccine into the ED_{50} of the reference vaccine, e.g.,

reference vaccine ED_{50} = 3.22 mg of brain tissue

test vaccine No. 1, ED_{50} = 1.07 mg of brain tissue

$$\text{antigenic value of vaccine No. 1} = \frac{3.22}{1.07} = 3.0$$

The challenge dose of virus is calculated from the results of challenge of the control mice. The results of the challenge are arranged in columns and a *Cumulative totals* column is obtained in a manner similar to that described above.

EXAMPLE

Dilution of virus	*Number of mice*	*Survived*	*Died*	*Cumulative totals Survived*	*Died*	*Percentage mortality*
$10^{-5.2}$	10	0	10	0	↑ 18	
$10^{-6.2}$	10	4	6	4	8	8/12=67
$10^{-7.2}$	10	8	2	↓ 12	2	2/14=14

[1] In making these calculations, the minus signs preceding the logarithms are disregarded.

Thus, the 50% end-point dilution lies between $10^{-6.2}$ and $10^{-7.2}$. Hence, using the same calculation as before:

$$\text{log of 50\% end-point dilution} = 6.2 + \frac{67 - 50}{67 - 14}$$

$$= 6.2 + \frac{17}{53}$$

$$= 6.2 + 0.32$$

$$= 6.52$$

Therefore the 50% end-point dilution is $10^{-6.52}$, i.e., the undiluted virus suspension contained $10^{6.52}$ LD_{50} or 3 310 000 LD_{50}. Since a $10^{-5.2}$ (1 : 158 000) dilution of virus was used as the challenge dose, that dose contained:

$$\frac{3\,310\,000}{158\,000} \text{ or } 21 \text{ } LD_{50}.$$

GUINEA-PIG TEST FOR CHICKEN-EMBRYO VACCINE

HILARY KOPROWSKI [1]

The test consists of the intramuscular inoculation of guinea-pigs with chicken-embryo vaccine, followed three weeks later by challenge of the animals with street or fixed virus of rabies.

Guinea-Pigs

Guinea-pigs weighing not less than 350 g at the beginning of the test should be chosen. The use of lighter animals is inadvisable.

Immunization Procedure

Each of 2 vials of vaccine representing each series or subseries in the final containers is reconstituted (irrespective of the manufacturer's recommended dosage) with sterile distilled water containing 2% of normal horse serum (3 ml of the diluent per dog dose of dried vaccine). Combine the two doses of the restored vaccine, withdraw a full dog dose (3 ml) and add this to 17 ml of distilled water containing 2% of horse serum. This will yield a 5% tissue suspension.

At least 10 healthy guinea-pigs are identified and inoculated into the gastrocnemius muscle on the inside of the leg as near to the nerve as possible with 0.25 ml of the diluted vaccine. At the same time, five or more healthy guinea-pigs are identified and set aside as vaccine controls.

Preparation of Challenge Material

Street rabies virus

Adult dogs are inoculated with 0.1 ml of a suspension of infected canine submaxillary-gland tissue kept frozen at —70°C. The injection is made bilaterally into the masseter muscle, using a 1-ml syringe and a 20-gauge,

[1] Director, Wistar Institute, Philadelphia, Penn., USA.

1-in (0.90-mm×25-mm) needle (see figure). When the animals die after showing signs of rabies, or when they are sacrificed when moribund, the submaxillary-gland tissue is removed, a small piece is separated by cutting with scissors, and the remainder is frozen in a large Petri dish and kept in a refrigerator at −50°C to −70°C. The selected portion is ground in a mortar with enough 10% normal rabbit serum in physiological salt solution to make a 10% suspension by weight. This suspension is then centrifuged in an angle-head centrifuge for one minute at 1000 rev/min and the supernatant liquid is separated. Tenfold dilutions of the supernatant liquid are made, using as diluent 10% normal rabbit serum in physiological salt solution and 0.03 ml is injected intracerebrally into Swiss albino mice 28-35 days old. These mice are observed for 21 days and the number of dead mice is recorded daily. After the period of observation, the LD_{50}

INOCULATION INTO MASSETER MUSCLE, USING DOG

By courtesy of Dr F. Perez-Gallardo, Madrid

titre of each preparation is calculated (for calculation see chapter 18, page 149).

In order to make a large virus pool, only those glands of which the titre exceeds $10^{-4.5}$ LD_{50} are pooled. The glands are removed from the freezer, thawed, cut into small pieces with scissors, and put into a Waring blendor with enough sterile 10% normal rabbit serum in physiological salt solution to make a 20% suspension by weight. The blendor is set in motion for 2 or 3 minutes only, in order to prevent heating of the material. It is then immersed in ice-water until well chilled, and the blending is again resumed for 2 or 3 minutes. The procedure is repeated four or five times, and the final preparation is filtered through one layer of gauze.

The filtered material is distributed in Pyrex-glass ampoules in 1-ml quantities. The ampoules are sealed and the material is shell-frozen in an alcohol-CO_2 bath. The ampoules are then stored at a temperature ranging from −50°C to −70°C.

Fixed rabies virus

The standard challenge virus (CVS) can also be used to challenge guinea-pigs. The preparation of the pools of virus in mice is described in chapter 18, page 145.

To increase the pathogenicity of this strain for guinea-pigs by intramuscular inoculation, a few intracerebral passages in young guinea-pigs (150-200 g) can be performed and a pool of guinea-pig-brain material used for challenge in place of mouse-brain material.

Challenge of the Guinea-Pigs

Three weeks after vaccination, the vaccinated guinea-pigs and controls are challenged by an intramuscular injection of canine street or fixed strain of rabies virus.

The dosage used is 0.5 ml of a suitable dilution of the virus suspension injected intramuscularly into the leg opposite that used for vaccination. The dilution is one two-fold dilution lower than that which kills 100% of the guinea-pigs in a preliminary titration ; therefore, if 100% of the guinea-pigs are killed with the 1 : 160 dilution, a 1 : 80 dilution should be used as the challenge virus for the potency test.

Interpretation of Results

A sample of results obtained with vaccines that have successfully passed potency tests is shown in the accompanying table. It will be seen that the vaccine has proven antigenic value for dogs challenged in this

RESULTS OF POTENCY TEST FOR CHICKEN-EMBRYO VACCINE

Batch of vaccine	LD_{50} titre in mice	Mortality ratio after intramuscular challenge with street virus								
		guinea-pigs							dogs	
		immunized with following dilutions of vaccine : [a]						non-vaccinated controls	vaccinated [b]	non-vaccinated controls
		1 : 5	1 : 20	1 : 80	1 : 320	1 : 1280	1 : 5120			
A	$10^{-2.75}$	0/8	0/9	0/6	0/10	0/8	—	10/10	0/5	3/6
B	$10^{-2.85}$	0/9	0/10	5/10	5/10	9/10	—	10/10	0/6 0/3 } two tests performed	3/5 10/10
C	$10^{-2.95}$	0/8	0/8	2/7	6/8	—	—	10/10	0/9 0/6 } two tests performed	8/9 6/10
D	$10^{-3.00}$	0/8	0/6	1/10	4/5	0/9	—	8/8	0/10	10/10
E	$10^{-3.90}$	—	—	1/4	1/6	4/5	0/4	8/9	0/6	18/25
F	$10^{-5.00}$	—	—	0/5	0/3	1/4	1/5	8/9	0/8	18/25
G	$10^{-3.90}$	—	3/15	—	—	—	—	13/16	0/7	3/5

[a] With batches A and D, 1 ml of 5% tissue suspension was used ; with all other batches, 0.5 ml was used. (Note: 0.25 ml of 5% tissue suspension is now used for routine testing.)

[b] 5 ml of 20% tissue suspension were used for vaccination.

laboratory with street virus. It may also be observed that in most instances dogs were protected against street-virus challenge with a vaccine preparation which immunized guinea-pigs in 5% chicken-embryo tissue suspension. As mentioned previously, the challenge virus should be of sufficient potency to cause the death of most if not all control animals. It has been observed in the past that the titre in mice must be $10^{-5.50}$ LD_{50} or higher for the challenge virus to meet this requirement.

In the actual test, 80% of the control animals should die of rabies and 70% of the vaccinated guinea-pigs should survive challenge inoculation without showing any symptoms of rabies. This test very closely parallels events occurring in nature and can be performed with relatively little labour. Only one inoculation with vaccine and one challenge inoculation are required. By employing locally isolated street-virus strain for challenge purposes, the test can be used to evaluate the potency of chicken-embryo vaccines in different geographical areas of the world against different strains of rabies. It has a disadvantage in that it requires dogs for the preparation of challenge virus. However, if a potent preparation of salivary-gland tissue is employed, a relatively small number of dogs will suffice to yield a preparation that can be used for a long period of time for challenge purposes.

Recently, tests with large batches of Flury LEP rabies vaccine in guinea-pigs using a CVS virus for challenge of vaccinated animals demonstrated that results of potency tests in guinea-pigs can be correlated with the intracerebral titration of the virus in mice. The paralysis pattern that is observed 4 to 10 days following the challenge correlates well with the virus titre of the vaccine and can therefore serve to facilitate the selection of potent products.

THE NYLAR TEST FOR MEASURING POTENCY OF ANTIRABIES VACCINE

DONALD J. DEAN [1]

The NYLAR[2] test for possible use in measuring the potency of modified live virus rabies vaccines is of the antigen-extinction type. Mice are vaccinated intraperitoneally with single injections of vaccine and immunity is challenged by virus injected intramuscularly instead of by the usual intracerebral route. Primary response to vaccine is measured, thus eliminating or minimizing the booster effect induced by multiple injections of vaccine or the challenge virus. A standard reference vaccine of adequate potency is required to eliminate the differences in response to antigen attributable to age, sex, or strain of mouse.

Groups of mice, 15 per dilution, are vaccinated intraperitoneally with 0.5 ml of 3 or more 5-fold dilutions of both test and reference vaccines. Ideally, dosage should be so chosen that protection in vaccinated mice after challenge will vary from 100 % in those given the greatest amount of vaccine to nil for those given the least. The immunity of all mice is challenged 21 days after vaccination by injecting infective mouse brain suspensions of the CVS strain of fixed virus intramuscularly in the thigh in an amount sufficient to kill not less than 50% of the mice given the smallest dose of reference vaccine ; mortality in controls should preferably be in the region of 100%.

The best results are obtained if the challenge virus has a titre of 10^{-6} or greater when titrated by the intracerebral route with a 0.03-ml dose in young adult mice. As required in the National Institutes of Health test, performance of the test vaccine must equal or exceed that of standard reference vaccine. Preliminary tests suggest that the NYLAR method may also be used to evaluate potency of vaccines containing inactivated virus. If vaccine potency does not permit multiple dilutions, a single dilution may be used.

Reference vaccine for this test should preferably be furnished by an official agency and should be carefully controlled for potency. For LEP vaccine, for example, a reference vaccine should have a mouse titre of not less than $10^{-3.5}$ when titrated by the intracerebral route in young adult mice and should meet the requirements of the guinea-pig protection test (see chapter 19, page 152).

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[1] Associate Director, Division of Laboratories and Research, New York State Department of Health, Albany.

[2] Abbreviation for " New York Laboratories and Research ".

PART IV

ANTIRABIES SERUM

CHAPTER 21

PRODUCTION OF THERAPEUTIC ANTIRABIES SERUM

PIERRE LÉPINE[1] *& PASCU ATANASIU*[2]

Institut Pasteur Method

A therapeutic antirabies serum is produced at the Institut Pasteur by immunization of horses or mules with the Pasteur strain of fixed rabies virus. Immunization is begun with inactivated Fermi-type vaccine, injection of which is continued for a period of two months. This is followed by a series of injections of increasing doses of live virus. All the injections are made subcutaneously. When the volume becomes too large for injection at a single site, multiple injections are made in different parts of the body.

The animals used must be carefully selected, as even for the same breed of horse the suitability of any particular animal for serum production varies according to its age, state of health, nutrition, and history. From time to time, samples of serum are taken from the animals undergoing immunization and the antibody titre is determined. Animals showing an inadequate response are eliminated and only those likely to yield a high-titre serum are retained.

The immunization schedule is as follows :

Day 1 to day 60 : 20 ml of Fermi-type vaccine every other day (30 injections).

Day 61 to day 72 : 4 injections, each of a quarter of a rabbit brain inoculated with fixed virus, at intervals of 3 days.

Day 73 to day 88 : 4 injections, each of half a rabbit brain inoculated with fixed virus, at intervals of 4 days.

Day 89 to day 98 : 2 injections, each of a whole rabbit brain inoculated with fixed virus, with an interval of 5 days.

Day 106 : first bleeding.

Day 136 : 1 injection of a whole rabbit brain inoculated with fixed virus.

Day 144 : second bleeding.

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[1] Head, Virus Division, Pasteur Institute, Paris, France.
[2] Virus Division, Pasteur Institute, Paris, France.

The immunization period thus lasts 98 days and the first bleeding is made 8 days later. A booster injection is given after another 30 days, followed by a second bleeding after 8 days. The booster injections and bleedings are repeated at these intervals, but the horses are allowed a rest period of 2 months each year.

Method of the Istituto Sieroterapico e Vaccinogeno, Siena

As originally described by d'Antona & Falchetti in the first edition of this monograph, the Siena method also makes use of horses for the production of a hyperimmune serum. Mirchamsy (1963), at the Razi Institute, Hessarek, Iran, has slightly modified the method for the immunization of adult mules.

The vaccine used by Mirchamsy is prepared from sheep inoculated intracerebrally with Sassari fixed virus and consists of a 5% brain-tissue suspension containing 0.5% of phenol and inactivated at 37°C for 24-48 hours. The mules used for production of the serum are simultaneously immunized against tetanus by several injections of tetanus toxoid adsorbed on aluminium phosphate (PATT).

The immunization schedule is as follows :

Day 1 : 20 ml of phenolized vaccine + 10 ml of PATT.
Day 2 to day 20 : 20 ml of phenolized vaccine daily (19 injections).
Day 21 : 30 ml of phenolized vaccine + 25 ml of PATT.
Day 22 to day 40 : 30 ml of phenolized vaccine daily (19 injections).
Day 41 : 45 ml of phenolized vaccine + 40 ml of PATT.
Day 42 to day 56 : 45 ml of phenolized vaccine daily (15 injections).
Day 57 to day 115 : resting period (60 days).
Day 116 : 30 ml of phenolized vaccine + 30 ml of PATT.
Day 117 to day 126 : 30 ml of phenolized vaccine daily (10 injections).
Day 127 : 45 ml of phenolized vaccine + 45 ml of PATT.
Day 128 to day 136 : 45 ml of phenolized vaccine daily (9 injections).
Day 137 : 30 ml of a 5% suspension of live virus (Sassari fixed strain) + 60 ml of PATT.
Day 138 to day 144 : 30 ml of a 5% suspension of live Sassari virus daily (6 injections).
Day 156 : first bleeding.

Accelerated Method

Fuenzalida & Palacios (1964) have developed an accelerated method of hyperimmunizing horses. The animals are first given a series of subcutaneous injections of vaccine in increasing concentrations and then simul-

taneous subcutaneous, intraperitoneal and intradermal injections of pure virus suspended in Freund's adjuvant without mycobacteria.

The horses are bled 20 days after the completion of the immunization schedule, which is as follows :

Day 1 : 40 ml of 1% inactivated vaccine subcutaneously.
Day 7 : 40 ml of 2% inactivated vaccine subcutaneously.
Day 14 : 40 ml of 5% inactivated vaccine subcutaneously.
Day 34 : 3 injections of an 8% suspension of live virus (brain tissue) in Freund's adjuvant — 5 ml intraperitoneally, 4 ml subcutaneously, 1 ml intradermally.
Day 41 : 3 injections of 16% live virus suspension in Freund's adjuvant — 5 ml intraperitoneally, 4 ml subcutaneously, 1 ml intradermally.
Day 48 : 3 injections of 20% live virus suspension in Freund's adjuvant — 8 ml intraperitoneally, 6 ml subcutaneously, 1 ml intradermally.
Day 68 : bleeding.

The vaccine used for the initial stages of immunization is prepared by the method previously described by Fuenzalida & Palacios (1955). Suckling mice are inoculated with fixed rabies virus and the vaccine prepared from the brain tissue is inactivated by irradiation with ultraviolet light. The live-virus suspensions are also prepared from the brains of suckling mice, with the addition of Freund's adjuvant without mycobacteria.

This rapid method seems to produce very satisfactory immunization and Fuenzalida & Palacios report neutralizing antibody titres that compare favourably with those obtained by other methods of immunization.

Immunization of Equines with Cultured Rabies Antigen

Antigens used

Two types of antigen—live and inactivated—are used. Both are prepared from the Pasteur strain of rabies virus cultured on baby-hamster-kidney (BHK_{21}) cells, clone 13, and maintained by weekly passages.

(*a*) *Inactivated antigen*

Cultures from the twentieth passage are pooled to give a total volume of about 10 litres. 0.03 ml of this pool usually gives a titre of $10^{-5.1}$ in the mouse and $10^{-5.5}$ in cell cultures.

The antigen is inactivated with betapropiolactone, using 0.1 ml for 1000 ml of antigen ; inactivation is confirmed by intracerebral inoculation of groups of 10 mice, all of which must survive.

(*b*) *Live antigen*

Cultures from the twentieth passage are pooled with an equal volume of cultures from the twenty-third passage. 0.03 ml of this pool usually gives a titre of $10^{-5.1}$ in the mouse and $10^{-5.5}$ in cell cultures.

Both antigens are stored in the frozen state at −30°C.

Immunization schedule

Either horses or mules may be used for immunization. Before commencing the immunization schedule, the animals are bled to test for possible cytopathogens in the serum. Provided that no cytopathogenic activity is demonstrated, immunization is carried out as follows :

First stage, using inactivated antigen

(*a*) Weekly intramuscular injections for a period of three weeks with the following doses :

1st injection	20 ml
2nd injection	50 ml
3rd injection	100 ml
4th injection	100 ml.

(*b*) Two intramuscular injections of 100 ml weekly for 3 weeks, i.e., a total of 6 injections, followed by the first control bleeding.

(*c*) Weekly intramuscular injections of 100 ml for two months, i.e., a total of 8 injections, followed by the second control bleeding.

Second stage, using live antigen

Injections once a week of the following doses :

1st injection	20 ml
2nd injection	50 ml
3rd injection	100 ml
4th injection	100 ml

followed by the third control bleeding.

This schedule is continued, with weekly injections of 100 ml and periodic bleedings, until a satisfactory titre is achieved.

Determination of protective titre

At each control bleeding, a steady rise in the antibody titre should be observed. Present experience indicates that the following titres can be expected at the first three bleedings.

First bleeding : The protective titre determined on cell cultures against 100 LD_{50} of virus should reach approximately 1 : 300, corresponding to about 30 IU/ml.

Second bleeding : The protective titre is determined in mice in comparison with the WHO reference serum. Using 100 LD_{50} of CVS, the reference serum should give a titre of about 1 : 2800 and the serum under test a titre above 1 : 3000, corresponding to more than 90 IU/ml. Using 300 LD_{50} of CVS, the reference serum should give a titre of approximately 1 : 1300 and the serum under test a titre of 1 : 2800, corresponding to about 170 IU/ml. When the titre is determined in cell cultures using 300 LD_{50} of virus, a value above 1 : 3000 should be obtained.

Third bleeding : The protective titre determined in mice infected with 300 LD_{50} of CVS should be about 1 : 6500, or 450 IU/ml.

Concentration and Purification of Antirabies Serum

The relatively large amounts of antirabies serum necessary for the protection of exposed persons, as well as the risk of anaphylactic accidents and other reactions, have led to the development of a number of methods for preparing a purified, concentrated serum.

Protein fractionation was first attempted by Habel (1945) using ammonium sulfate and various other methods have since been described.[1] The method of fractionation and purification adopted at the Institut Pasteur consists of two stages : first, enzymatic digestion of the proteins followed by precipitation with ammonium sulfate ; second, removal of the excess proteins by thermo-coagulation.

Whichever method is adopted, it is advisable to determine the final protein content of the purified serum and relate this to its protective titre (see chapter 22, page 167). Paper electrophoresis should also be performed to check the fractionation of the proteins. In general, a concentrated purified serum with a titre of 20 IU/ml should not contain more than 5% of total serum proteins.

Antirabies Human Gamma-Globulin

The production of specific antirabies human gamma-globulin, which would avoid the adverse reactions encountered when heterologous serum is administered, appears to be a practicable procedure, although expensive (Sikes et al., to be published). For the time being, however, production is still in the experimental stage.

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[1] See Koprowski et al. (1950), Pope & Stevens (1951), Delsal & Mirchamsy (1953), Devi et al. (1956), Wang & Lin (1957).

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QUANTITATIVE ASSAY AND POTENCY TEST OF ANTIRABIES SERUM

PASCU ATANASIU [1]

Principle

The method described below consists in neutralizing a constant dose of the previously titrated challenge virus with a series of different dilutions of the serum. The method is used mainly for the assay and potency testing of therapeutic antirabies serum, but it is also applicable to any serum containing rabies antibody. Thus, it can be used to determine the antibody titres of human sera collected during therapeutic trials of different vaccines.

The method comprises the following three stages :

1. Preparation and titration of the challenge virus.

2. Serum-virus neutralization—preparation of the serum, and of the serum-virus mixtures ; inoculation of mice.

3. Interpretation of the results.

1. Preparation and Titration of Challenge Virus

Challenge virus

The strain normally used is the standard challenge virus strain (CVS), as used in the mouse potency test for rabies vaccine (see chapter 18, page 145). The laboratory strain of fixed virus may also be used, provided that its LD_{50} for the mouse is known and remains constant.

Titration of stock virus

The challenge virus is stored as a 20% suspension, which is dispensed into ampoules and deep frozen. When carrying out the test, an ampoule is taken from the stock and thawed rapidly under the tap. Serial tenfold dilutions are then prepared, giving concentrations of 2×10^{-2}, 2×10^{-3} etc., up to 2×10^{-7}. The diluent used is twice-distilled water, to which has been added 2% of normal horse serum, inactivated for 30 minutes at 56°C.

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[1] Virus Division, Pasteur Institute, Paris, France.

In each of five haemolysis tubes is then placed 0.5 ml of one of the virus dilutions, starting with 2×10^{-3} and ending with 2×10^{-7}. To each tube is then added 0.5 ml of inactivated horse serum diluted 1 : 5, so that the final dilutions in the four tubes are 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}.

After shaking, the mixtures are incubated for 1½ hours at 37°C, in order to ensure that the conditions are the same as those of the serum-virus neutralization stage. The tubes are then cooled in ice-water. Mice are inoculated intracerebrally with 0.03 ml of the virus suspension, five mice being used for each dilution. Provided that the highest dilution is used first, all the inoculations may be made with the same syringe. A record is made of the number of mice that die between the 6th and 20th day after inoculation.

Calculation of virus titre

The calculation is made by the Reed & Muench method, as shown in the following example :

Virus dilution	*Deaths*	*Survivals*	*Cumulative totals*		*Percentage mortality*
			Deaths	*Survivals*	
10^{-3}	5	0	↑ 17	0	
10^{-4}	5	0	12	0	
10^{-5}	5	0	7	0	100
10^{-6}	2	3	2	3	40
10^{-7}	0	5	0	8 ↓	

The following formula is then applied :

$$\frac{50-(\%\text{ mortality next below 50})}{(\%\text{ mortality next above 50})-(\%\text{ mortality next below 50})}$$

Substituting the figures from the above example, this gives :

$$\frac{50-40}{100-40}=\frac{10}{60}=0.16.$$

Since the logarithm of the dilution factor (10) is 1, it follows that this figure (0.16) represents the difference between the log of the 50% end-point dilution and the log of the next lower dilution (10^{-6}). The log of the 50% end-point dilution is thus $0.16-6=-5.84$, and the 50% end-point dilution is $10^{-5.84}$.

This dilution of the stock virus will contain 1 LD_{50} in 0.03 ml and it is now possible to calculate the dilution of virus that will be needed for titration of antirabies sera. Since 300 LD_{50} are used for the titration, the virus dilution required will be

$$300\times10^{-5.84}=\frac{300}{692\,000}\text{ or approximately }\frac{1}{2\,300}$$

2. Serum-Virus Neutralization

Inactivation

The sera to be tested are inactivated for 30 minutes at 56°C.

Neutralization

The following serial dilutions of the serum under test are prepared: 1 : 500, 1 : 1000, 1 : 2000 and 1 : 4000.[1] From each of these dilutions, 0.5 ml is transferred to one of a series of test tubes. Next, 0.5 ml of the virus dilution corresponding to 300 LD_{50} (in the example given above, a dilution of 1 : 2300) is added to each tube. This results in a twofold dilution of both the virus and the serum, so that the final virus dilution is 1 : 4600, or $10^{-3.66}$, and the final serum dilutions are 1 : 1000, 1 : 2000, 1 : 4000 and 1 : 8000.

It is advisable to include in the test a reference serum which is titrated at the same time as the unknown sera. This reference serum, or standard, is a serum of known titre which has previously been titrated against the international standard and then stored in the laboratory with the usual precautions. If the results of the test are to be expressed in international units (IU), the inclusion of such a reference serum is mandatory.

Titration of virus control

It is essential to determine the actual quantity of virus used in the test. For this purpose, serial tenfold dilutions are made from the 1 : 2300 dilution of the stock virus, calculated to contain 300 LD_{50}/0.03 ml. In this way dilutions of $1 : 23 \times 10^3$, $1 : 23 \times 10^4$, $1 : 23 \times 10^5$ and $1 : 23 \times 10^6$ are obtained. To 0.5 ml of each of these dilutions, 0.5 ml of inactivated, normal horse serum, diluted 1 : 5, is added, giving the following final dilutions of virus:

$1 : 46 \times 10^3$ or $10^{-4.66}$
$1 : 46 \times 10^4$ or $10^{-5.66}$
$1 : 46 \times 10^5$ or $10^{-6.66}$
$1 : 46 \times 10^6$ or $10^{-7.66}$

After shaking, the tubes containing the sera under test and the control tubes are left in the incubator for 1½ hours at 37°C.

Inoculation of mice

After incubation at 37°C for 1½ hours, all the tubes are placed in a vessel filled with ice-water. Batches of mice weighing 14 g to 16 g each are

[1] If the levels of antibody are expected to be low, as in vaccinated human subjects, serum dilutions starting with 1 : 5, 1 : 25, 1 : 125 are employed and the final virus dilution should be 20-50 LD_{50}.

then inoculated intracerebrally with 0.03 ml of each dilution, using 5 mice per dilution. This is done both for the sera under test and for the virus control. When a large number of sera are being tested, it is important to make the injections of the virus control after having injected half the sera ; this ensures that the dilutions of the virus control and the sera under test are kept for the same average length of time before making the injections. The different groups of mice are placed separately in labelled jars and kept under observation ; a record is made of those dying between the 6th and 20th day.

3. Calculation and Interpretation of Results

The following is a typical protocol, based on the titration of an experimental batch of therapeutic serum with dilutions of the stock virus referred to in the example on page 168.

Determination of the number of LD_{50} actually used in the experiment

Virus dilution	*Deaths*	*Survivals*	*Cumulative totals*		*Percentage mortality*
			Deaths	*Survivals*	
$10^{-4.66}$	5	0	↑ 10	0	
$10^{-5.66}$	5	0	5	0	100
$10^{-6.66}$	0	5	0	5	0
$10^{-7.66}$	0	5	0	10 ↓	

Applying the formula for calculation of the 50% end-point dilution :

$$\frac{50 - 0}{100 - 0} = 0.50$$

whence the log of the 50% end-point dilution $= 0.50 - 6.66 = -6.16$.

Thus, a virus dilution of $10^{-6.16}$ would contain 1 LD_{50} in 0.03 ml. The final virus dilution used for neutralization was 1 : 4600 or $10^{-3.66}$ (see page 169). Therefore the number of LD_{50} actually used in the test was

$$\frac{10^{-3.66}}{10^{-6.16}} = 10^{2.50} = 316 \text{ (antilog of 2.50).}$$

Calculation of the ED_{50} of the serum under test

Serum dilution	*Deaths*	*Survivals*	*Cumulative totals*		*Percentage mortality*
			Deaths	*Survivals*	
1 : 1000	1	4	1	6 ↑	14
1 : 2000	3	2	4	2	66
1 : 4000	4	0	8	0	100
1 : 8000	5	0	↓ 13	0	

Applying the formula for calculation of the 50% end-point dilution :

$$\frac{50 - 14}{66 - 14} = \frac{36}{52} = 0.69.$$

Since the logarithm of the dilution factor is 0.301, the difference between the logarithm of the 50% end-point dilution and the logarithm of the next lower dilution is :

$$0.69 \times 0.301 = 0.208.$$

Hence, the logarithm of the 50% end-point dilution is 3.208 and the 50% end-point dilution is $10^{-3.208}$ or 1 : 1900.

Calculation of the ED_{50} of the reference serum

Serum dilution	*Deaths*	*Survivals*	*Cumulative totals*		*Percentage mortality*
			Deaths	*Survivals*	
1 : 1000	1	4	1	6	14
1 : 2000	4	1	5	2	71
1 : 4000	5	0	10	1	100
1 : 8000	4	1	14	1	

Applying the formula once more :

$$\frac{50 - 14}{71 - 14} = \frac{36}{57} = 0.63$$

and $0.63 \times 0.301 = 0.189$.

Thus, the 50% end-point dilution is $10^{-3.189}$.

The number of international units (IU) contained in the reference serum is fixed arbitrarily at 80 per ml. To express the potency of a serum under test in IU, its neutralizing power must be compared with that of the reference serum. This is done by calculating the difference between the logarithms of the 50% end-point dilutions of the two sera. In the above example, this gives :

$$-3.19 - (-3.21) = 0.02.$$

The serum under test is thus $10^{0.02} = 1.05$ times as potent as the reference serum. Therefore, its titre is $1.05 \times 80 = 84$ IU.

Therapeutic potency test

An antirabies serum should satisfy the requirements laid down in the fourth report of the WHO Expert Committee on Rabies : [1]

[1] *Wld Hlth Org. techn. Rep. Ser.*, 1960, **201**, 10.

" A serum shall pass the test for sufficient therapeutic potency if, in a single comparative assay, it is revealed to be equal to or better than the International Standard Serum. In case a serum fails the test, two more similar tests may be carried out. If the serum proves equal to or better than the International Standard Serum in both these additional tests, it shall pass. The outcome is ' equal ' or ' better ' if the total survivor fraction (survivors/total number of mice) for the serum under test is equal to or larger than that for the standard. "

PROPAGATION OF RABIES VIRUS IN TISSUE CULTURE

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Almost as soon as tissue culture techniques were developed, they were applied to the study of rabies virus. Levaditi (1913, 1914) reported the first successful propagation of rabies virus in spinal ganglia maintained in a medium containing coagulated monkey plasma. Stoel (1930) and Webster & Clow (1936) carried a strain of the virus through several passages in a suspension of chicken or mouse embryo brain without loss of infectivity.

The study of rabies virus in tissue culture is extremely important, not only as a tool for obtaining knowledge about the virus itself but also as a possible method for the production of large quantities of rabies virus for the preparation of vaccine.

Until recently, however, studies with primary cell explants did not produce much information about the kinetics of virus replication. The development of several cell lines and cell strains that are susceptible to rabies virus infection and the application of new laboratory techniques, such as immunofluorescent antibody staining, for detection of viral antigen within infected cells have led to a better understanding of the mechanism of rabies infection.

The adaptation of several strains of rabies virus to different tissue culture systems opened the way to a series of investigations on the mechanism of cell lysis observed in some systems, on establishment of an endosymbiotic relationship between cells and virus in other systems, on observation of rabies virus under the electron microscope, and on determination of the chemical composition of intracytoplasmic inclusions observed in rabies-infected tissue-culture cells. Recent developments also seem to indicate that rabies virus propagated in various tissue culture systems can be used either as a live attenuated vaccine or as an inactivated vaccine for immunization of animals and in one case, using human diploid cell strain (HDCS), for immunization of man.

It is impossible to discuss here all aspects of the various tissue culture techniques; only those aspects directly related to study of rabies virus will be described.

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Tissue Culture Systems

1. *Primary cultures*

(*a*) *Chicken fibroblasts.* While primary embryonic chicken fibroblasts are very susceptible to rabies virus and many strains of fixed and street virus have been propagated in these cells, most embryonated eggs are infected with other viruses—primarily of the lymphomatosis group—and may also carry mycoplasma. The latent contaminants can interfere with the growth of rabies virus and produce inconsistent results. Despite this difficulty, Kaplan et al. (1960) demonstrated that rabies virus in chick embryo fibroblasts prevents plaque formation by western equine encephalomyelitis virus, and Habel [1] succeeded in maintaining rabies virus in chicken fibroblasts through numerous serial passages.

LEP and HEP Flury virus grown in this system was experimentally used for immunization of dogs by Dean et al. (1964) and for immunization of man by Ruegsegger (1962).

(*b*) *Kidney-cell cultures.* Susceptibility of mouse kidney-cell cultures to infection with rabies virus was reported by Vieuchange et al. (1956). Subsequently, Kissling (1958) was able to make serial passages of both street and fixed virus in hamster kidney culture. Fenje (1960) made similar observations and his strain was adapted to pig kidney cultures (Abelseth, 1964a). The two hamster-kidney-adapted strains were found to be antigenic for experimental animals (Kissling & Reese, 1963 ; Abelseth, 1964b).

(*c*) *Dog salivary glands.* Depoux (1963, 1964) demonstrated the ability of dog salivary glands maintained in tissue culture to support the growth of rabies virus. A cytopathic effect was reported in this system after about 20 passages. A chronic infection of the cells could also be obtained.

2. *Continuous cell lines*

Several stable cell lines support the growth of rabies virus. Stable tissue culture systems are preferable to primary cultures since they can be propagated easily, characterized to determine that they are free of contaminants and stored in a frozen state for long-term use, but virus propagated in these lines cannot be used for immunization of man.

(*a*) *Mice ependymoma cell line.* This cell line was used by Atanasiu & Laurent (1957) for cultivation of rabies virus. It was reported that after passages of street and fixed rabies virus, an inconsistent cytopathic

[1] Unpublished communication to WHO.

effect was observed and that intracytoplasmic inclusions resembling Negri bodies were seen.

(*b*) *BHK-21 line of hamster kidney fibroblasts*. Rabies virus adapted to grow in these cells (Atanasiu et al., 1963 ; Fernandes et al., 1963) was used for purification studies and for the determination of the size and shape of particles by electron microscopy. The biochemistry of the intracytoplasmic inclusions present in the infected cells was studied (Love et al., 1964).

(*c*) *Rabbit endothelium cell line*. Rabies virus could be propagated in this line for a prolonged, if not indefinite, period, without interfering with the mechanism of cellular replication (Fernandes et al., 1964), even though all cells were infected and those cells undergoing division showed the presence of viral antigen throughout the mitotic process. Antirabies serum did not interfere with cell-to-cell transmission of the virus during mitosis. When exposed to antirabies serum and complement, infected cells underwent complete lysis.

(*d*) *Dog kidney cell line*. An epithelial line of dog kidney cells effectively supported the growth of different strains of rabies virus (Wiktor, unpublished data). A chronic infection could be established with only some of the cells showing the presence of specific fluorescing inclusions. Like HDCS (see below), the dog kidney cell line produced a large amount of virus, and the infected culture could be carried by regular splitting for numerous passages. Flury LEP and HEP strains grown in these cells proved to be good antigens when tested in guinea-pigs and mice.

3. *Cell strains*

Human diploid cell strain (HDCS). Various strains of rabies virus have been adapted for growth in this cell system (Hayflick & Moorhead, 1961) by means of the cell mixing technique (see below).

The HEP-adapted virus produced a cytopathic effect in the HDCS and during the course of its propagation became more infective for other tissue-culture systems. Both live and inactivated vaccines prepared in the diploid cell strains have been found highly antigenic for mice, guinea-pigs and monkeys (Wiktor et al., 1964).

Techniques

Adaptation of rabies virus to growth in different cell systems

Observations on HDCS and several other cell lines reveal that infection of monolayers or dispersed cell cultures induces fluorescence of a very small number of cells, and that serial transfer of either the supernatant

medium or cell extracts in a homologous tissue culture system often results in a gradual decrease of infection. As a rule, after a few passages, the virus is completely lost.

Infected cultures, maintained in an actively growing state by regular trypsinization and division every 3 to 4 days when cell sheets become confluent, show a gradual increase in the number of fluorescing cells. After a few such divisions (from 4 to 10, depending on the cell system and the virus strain), all cells show fluorescence and the presence of intracytoplasmic inclusions.

After a certain number of subcultures in some tissues, like HDCS and the BHK-21 line, the cells stop dividing and virus passage can be effected only by mixing a portion of infected cells with a non-infected homologous cell population at each transfer passage.

In other cell systems, like rabbit endothelium and dog kidney cells, no cytopathic effects are observed and an endosymbiotic or chronic state of infection can be maintained without the addition of new cells.

In most cases, after about 40 passages, the virus is sufficiently adapted to grow in a given cell system and can be propagated by using either the supernatant medium or cell extracts for infection of new cells. The presence of cell debris, even after ultrasonic treatment (sonication), facilitates infection of new cells.

Titration of virus in tissue culture systems

Although different techniques can be employed to detect virus in infected tissue cultures, mouse inoculation remains the usual method of quantitative determination of virus titre. Presence of the virus can be assayed using either the supernatant medium or whole culture (medium and cells). In the latter case, cells are detached from the glass surface by a rubber policeman, and the culture is frozen and thawed several times to release the virus.

In order to detect viral antigen by immunofluorescent staining and by the presence of intracytoplasmic inclusions and cytopathic effects, cells should be grown in Petri dishes or tubes containing coverslips. Since cytopathic effects in rabies-infected tissue culture cannot be produced regularly, it is impossible to titrate the virus by direct morphological observation of the infected culture. Staining of the infected cells by fluorescing antibodies permits a more exact determination of infectivity end-points. The technique of the test is as follows : Serial dilutions of virus are prepared in tissue culture medium and an aliquot of each dilution (0.5 ml) is mixed with 5 ml of the cell suspension and poured into Petri dishes containing coverslips. The concentration of cells is adjusted so that a confluent monolayer is obtained after 3 to 4 days of incubation. At the end of the incubation period (5 to 6 days after infection), coverslips are removed and stained

with fluorescing antibody conjugate.[1] The highest dilution of virus causing fluorescence of cells is considered the titration end-point.

Staining of intracytoplasmic inclusions

Although general techniques used for staining of Negri bodies (see chapter 4, page 51) can be used for the detection of intracytoplasmic inclusions in tissue-culture preparations, staining with May-Grunwald-Giemsa stain was found to give more consistent results.

Preparations are washed in phosphate buffer saline (PBS) for 2 minutes, then fixed in Bouin fixative for 20 minutes. After being washed in 70% ethanol for 20 minutes, they are stained with May-Grunwald stain for 15 minutes. The May-Grunwald stain is then removed and the preparations are covered with Giemsa stain (1 : 10) for 30 minutes. If necessary, the preparations are differentiated in a 0.5% solution of acetic acid for a few seconds. They are then washed with water and treated successively with acetone, a mixture of acetone and xylol, and pure xylol, allowing 2 minutes in each solvent. Finally, they are mounted on slides with Canada balsam.

Interference test

Rabies-infected cultures showing a high percentage of specifically fluorescing cells are resistant to reinfection with other strains of rabies virus and non-related plaque-forming viruses, such as polio, EEE, WEE, VSV, and Mengo. The following technique is recommended for demonstration of the interference phenomenon against a plaque-forming virus (Kaplan et al., 1960 ; Wiktor et al., 1964).

Infected cells are grown in Petri dishes, the medium is removed and the cells are washed in PBS when the culture is completely sheeted, usually 3 to 4 days after preparation. Challenge virus is added to the monolayer (0.2 ml per Petri dish) at appropriate dilutions to produce approximately 100 plaques per Petri dish. After 1 hour of adsorption at 37°C the cells are washed again with PBS, agar nutrient is added, and the cells are incubated at 37°C for 3 to 5 days, depending on the challenge virus used.

After incubation, 4 ml of a 1 : 10 000 dilution of neutral red is added and the plaques are counted 4 hours later. This count is compared with the number of plaques produced in a culture that was not infected with rabies virus before addition of the challenge virus.

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[1] The technique described in chapter 5, page 59, can be used for immunoflorescent antibody staining of tissue culture preparations. Coverslips with infected cells are removed and placed in the staining racks, where they are rapidly washed in PBS and fixed in cold acetone (−20°C) for 30 minutes. After fixation they are dried and placed in individual Petri dishes and covered with a few drops of conjugate. The preparations are incubated for 40 minutes at 37°C and then washed for 5 minutes in two changes of PBS and one change of distilled water, dried and mounted in Elvanol (polyvinyl alcohol) semipermanent mounting medium. Control preparations, including non-infected cultures and an infected culture stained with the conjugate previously absorbed to a suspension of rabies-infected mouse brain, should be included in the test.

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